

**INDIAN AGRICULTURAL  
RESEARCH INSTITUTE LIBRARY,  
NEW DELHI.**







**PROCEEDINGS**  
**OF THE**  
**ROYAL SOCIETY OF LONDON**  
**SERIES B**  
**CONTAINING PAPERS OF A BIOLOGICAL CHARACTER**  
  
**VOL. CVII.**

**LONDON:**  
**PRINTED FOR THE ROYAL SOCIETY AND SOLD BY**  
**HARRISON AND SONS, LTD., ST. MARTIN'S LANE,**  
**PRINTERS IN ORDINARY TO HIS MAJESTY.**

LONDON :

HARRISON AND SONS, LTD., PRINTERS IN ORDINARY TO HIS MAJESTY,  
ST. MARTIN'S LANE.

# CONTENTS.

## SERIES B. VOL. CVII.

No. 748—September 3, 1930.

PAGE

The Relationship of Climatic and Geological Factors to the Composition of Soil Clay and the Distribution of Soil Types. By E. M. Crowther. Communicated by Sir John Russell, F.R.S.....	1
The Relation between the Anterior Pituitary Body and the Gonads. Part I.—The Factors concerned in the Formation of the Corpus Luteum. By M. Hill and A. S. Parkes. Communicated by C. L. Evans, F.R.S. (Plate 1.).....	30
The Relation between the Anterior Pituitary Body and the Gonads. Part II.—The Induction of Ovulation in the Ancestrous Ferret. By M. Hill and A. S. Parkes. Communicated by C. L. Evans, F.R.S. (Plates 2-4.).....	39
A Cytological Demonstration of "Genetic" Crossing-Over. By C. D. Darlington. Communicated by Sir Daniel Hall, F.R.S. (Plate 5.).....	50
The Development and Vascularisation of the Corpus Luteum in the Mouse and Rabbit. By R. Deanesly. Communicated by J. P. Hill, F.R.S. (Plates 6-8.)...	60
The Distillation of Vitamin D. By F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. G. C. Jenkins and T. A. Webster. Communicated by H. H. Dale, Sec. R.S.	76
The Effects of Further Irradiation of the Radiation Products of Ergosterol. By F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. G. C. Jenkins and T. A. Webster. Communicated by H. H. Dale, Sec. R.S. ....	91
The Vital Staining of Normal and Malignant Cells. III.—Vital Staining of Acinar Cells of the Pancreas, and its Bearing on the Theories of Vital Staining with Basic Dyes. By R. J. Ludford. Communicated by J. A. Murray, F.R.S. (Plate 9.).....	101

No. 749.—October 1, 1930.

The Heat Production in Isometric and Isotonic Twitches. By A. V. Hill, F.R.S....	115
Observations on the Living Eggs of the Rabbit. By G. Pineus. Communicated by F. H. A. Marshall, F.R.S. (Plates 10-13.) .....	132
Cell Structure and Cell Activity. By M. Penrose and J. H. Quastel. Communicated by Sir Frederick Hopkins, F.R.S. ....	168

The Frozen State in Mammalian Muscle. By T. Moran. Communicated by Sir William Hardy, F.R.S.....	182
The Functions of the Corpus Luteum.—IV., The Relation of Oestrin to the Luteal Phase of the Oestrous Cycle. By A. S. Purkes. Communicated by C. L. Evans, F.R.S. (Plates 14–16.) .....	186

No. 750.—November 3, 1930.

Cellular Individuality in the Higher Animals, with Special Reference to the Individuality of the Red Blood Corpuscle.—II. By C. Todd, F.R.S. ....	197
A Method for the Estimation of Iron in Biological Material. By R. Hill. Communicated by Sir Frederick Hopkins, F.R.S. ....	205
The Heat of Rigor of Mammalian Muscle. By E. C. Smith. Communicated by Sir William Hardy, F.R.S.....	214
Observations on Certain Physiological Processes of the Marmot. I.—The Heart. By G. Endres, B. H. C. Matthews, H. Taylor and A. Dale. Communicated by J. Barcroft, F.R.S. (Plates 17 and 18.) .....	222
Observations on Certain Physiological Processes of the Marmot. II.—The Respiration. By G. Endres and H. Taylor. Communicated by J. Barcroft, F.R.S. ...	231
Observations on Certain Physiological Processes of the Marmot.—III, IV and V. By G. Endres. Communicated by J. Barcroft, F.R.S. ....	241
The Accumulation of Electrolytes in Plant Cells—A Suggested Mechanism. By G. E. Briggs. Communicated by F. F. Blackman, F.R.S. ....	248
The Scattering of Light in Protein Solutions. I.—Gelatin Solutions and Gels. By K. Krishnamurti. (Abstract.) Communicated by F. G. Donnan, F.R.S.....	269

No. 751.—December 2, 1930.

Prothrombase—Its Preparation and Properties. By J. Mellanby, F.R.S.....	271
The Porphyrin of Component c of Cytochrome and its Relationship to other Porphyrins. By R. Hill and D. Keilin, F.R.S. ....	286
The Differential Action of X-Rays on Tissue, Growth and Vitality. Part II.—The Biological Reaction to X-Radiation in Relation to a Phenomenon termed Antagonism. By W. Moppett. Communicated by J. T. Wilson, F.R.S. ....	293
The Differential Action of X-Rays on Tissue, Growth and Vitality. Part III.—The Biological Reaction to X-Radiation in Relation to the Area of Tissue Irradiated. By W. Moppett. Communicated by J. T. Wilson, F.R.S. (Plate 19.) .....	302
The Differential Action of X-Rays on Tissue, Growth and Vitality. Part IV.—The Biological Reaction to X-Radiation in Relation to Time. By W. Moppett. Communicated by J. T. Wilson, F.R.S. ....	308

	PAGE
The Mechanism of Ciliary Movement.—VI. Photographic and Stroboscopic Analysis of Ciliary Movement. By J. Gray, F.R.S. (Plates 20-22.).....	313
The Contraction of the Extrinsic Muscles of the Eye by Choline and Nicotine. By W. S. Duke-Elder and P. M. Duke-Elder. Communicated by C. L. Evans, F.R.S. ....	332
Secondary Electronic Emissions from Metal Foils and Animal Tissues. (Extract and Summary.) By W. V. Mayneord. Communicated by H. Hartridge, F.R.S. ....	344

## No. 752.—January 1, 1931.

Address of the President, Sir Ernest Rutherford, O.M., at the Anniversary Meeting, December 1, 1930 .....	348
The Isolation by Cataphoresis of Two Different Oxyhaemoglobins from the Blood of some Animals. By A. Geiger. Communicated by H. H. Dale, Sec. R.S. ....	368
The Origin of the Mesoderm in the Gastropod <i>Viviparus</i> ( = Paludina). By W. Fernando. Communicated by E. W. MacBride, F.R.S. (Plates 23-28.).....	381
The Origin and Development of the Pericardium and Kidneys in <i>Ostrea</i> . By W. Fernando. Communicated by E. W. MacBride, F.R.S. (Plates 29-32.) .....	391
Croonian Lecture.—The Theories of the Bacteriophage. By J. Bordet, For. Mem. R.S. ....	398

## No. 753.—February 3, 1931.

Studies on Echinoderm Oogenesis.—I. <i>Antedon bifida</i> (Pennant). By L. A. Harvey. Communicated by J. H. Ashworth, F.R.S. ....	417
Studies on Echinoderm Oogenesis.—II. <i>Asterias rubens</i> , Linné. By L. A. Harvey. Communicated by J. H. Ashworth, F.R.S. ....	441
The Relation between the Anterior Pituitary Body and the Gonads. Part III.—Fractionation and Dilution of Ovary-Stimulating Extracts. By M. Hill and A. S. Parkes. Communicated by C. L. Evans, F.R.S. (Plates 33 and 34.).....	455
The Pupillary Mechanism of the Teleostean Fish <i>Uranoscopus scaber</i> . By J. Z. Young. Communicated by E. S. Goodrich, F.R.S. ....	464
Metabolic Changes associated with Pigmentary Effector Activity and Pituitary Removal in <i>Xenopus laevis</i> .—I. Respiratory Exchange. By E. Charles. Communicated by H. H. Dale, Sec. R.S. ....	486
Metabolic Changes associated with Pigmentary Effector Activity and Pituitary Removal in <i>Xenopus laevis</i> .—II. Calcium and Magnesium Content of the Serum. By E. Charles. Communicated by H. H. Dale, Sec. R.S. ....	504

No. 754.—March 7, 1931.

	PAGE
Studies on the Flexor Reflex.—I. Latent Period. By J. C. Eccles and Sir Charles Sherrington, F.R.S. (Plates 35 and 36.) .....	511
Studies on the Flexor Reflex.—II. The Reflex Response Evoked by Two Centripetal Volleys. By J. C. Eccles and Sir Charles Sherrington, F.R.S. (Plates 37 and 38.) .....	535
Studies on the Flexor Reflex.—III. The Central Effects Produced by an Antidromic Volley. By J. C. Eccles. Communicated by Sir Charles Sherrington, F.R.S. (Plates 39 and 40.) .....	557
Studies on the Flexor Reflex.—IV. After-Discharge. By J. C. Eccles and Sir Charles Sherrington, F.R.S. (Plates 41 and 42.) .....	586
Studies on the Flexor Reflex.—V. General Conclusions. By J. C. Eccles and Sir Charles Sherrington, F.R.S. ....	597
The Osmotic Changes in Some Marine Animals. By R. Margaria. Communicated by A. V. Hill, F.R.S. ....	606

## OBITUARY NOTICES.

Gadow, Hans Friedrich .....	i
Balfour, The Earl of .....	iv
Dawkins, Sir William Boyd .....	xxjii
Evans, John William .....	xxvii
Index .....	xxxix

# PROCEEDINGS OF THE ROYAL SOCIETY.

## SECTION B.—BIOLOGICAL SCIENCES.

631.48 : 561.5

### *The Relationship of Climatic and Geological Factors to the Composition of Soil Clay and the Distribution of Soil Types.*

By EDWARD M. CROWTHER, Rothamsted Experimental Station.

(Communicated by Sir John Russell, F.R.S.—Received June 3, 1930.)

Few soil problems have aroused more discussion and controversy than the assessment of the relative importance of climatic and geological factors in soil formation. At International Soil Congresses such as that held in 1927 in the United States it has been apparent that British and other workers familiar with small highly cultivated areas of irregular topography and varied geology attach much less importance to the climatic factors than do the Russians whose experience is largely of vast plains of fairly uniform loess material extending over well defined climatic zones. Purely practical considerations led under these extreme conditions to the development of geological and climatic systems of soil classification respectively, but both systems were found to require considerable modification when they were applied to other countries or when the scale of soil mapping was greatly changed. Even in the British Isles a generalised soil map would show the influence of climate, and detailed soil mapping by the Russian school would allow for considerable modifications of the climatic soil types by variations in local geology and topography.

Both systems are open, however, to the more fundamental criticism that they are based not on the actual properties of the objects classified but on external factors which have influenced the formation of the soil to varying and unknown degrees. The Russian work has demonstrated that an essential preliminary to all field and laboratory examination of soils should be the



recognition and separation of the soil profile down to the unaltered parent material into a series of distinct horizons of approximately uniform composition and mode of formation. These morphological studies, however, have not led to any system of soil classification capable of general application largely because they yield essentially qualitative and highly subjective information. There is, therefore, a great need at present time for the combination of exact information on the composition of soils on a profile basis with such mapping of similar soils as will enable the effects of geological, climatic, and human influences to be deduced by a rigid analysis of quantitative data. The present paper contains a preliminary attempt (1) to separate the influence of two climatic factors and a broad geological grouping on one of the most fundamental chemical characteristics of soils, the molecular ratio of silica to alumina in the clay fraction and (2) to examine the contribution of mean annual rainfall and temperature to soil formation by an examination of the distribution of recognised soil types. The method of analysis is applicable to other geographical and ecological problems in which it is necessary to assess the importance of several contributory factors some of which may be quantitative and others qualitative.

### *The Composition of Soil Clay.*

Chemical and physical analyses of soils have in the past contributed relatively little to our knowledge of the processes involved in the formation of soils and in the maintenance of their fertility. The failure may be ascribed partly to an excessive attention to the elements believed to have direct nutritive value to plants and partly to the separation of chemical from physical analyses. E. W. Hilgard's (1905) numerous comparisons of the acid extracts of soils from arid and humid regions demonstrated little beyond the accumulation of the commoner bases and acids near the surface in dry regions and of the finer soil particles in the subsoil in the humid districts. Although it has always been customary to discard as stones any particles larger than 2 or 3 mm. in diameter before analysing a soil, the desirability of making a physical fractionation at a much lower value of particle size has only recently been recognised in soil laboratories. It is apparently not yet appreciated by ceramic chemists and sedimentary petrologists, although they too have come to distrust the earlier attempts to distinguish by means of acid extractions or "rational analysis" between the essential product of weathering, and the coarser slightly weathered or adventitious material. Total analyses have now generally replaced acid extractions of soils but much of the information they provide

can be obtained more directly by simple physical analysis. Thus an accumulation of aluminium in a B horizon may arise from a purely mechanical washing down of fine particles. A combination of physical fractionation with chemical and mineralogical analyses of the fractions is desirable, but for many purposes a chemical analysis of the finest fraction of mechanical analysis proves sufficient. The precise value to be set as the upper limit of particle size for the weathering complex or colloidal material is difficult to determine but a compromise between theoretical requirements and laboratory convenience is afforded by the definition of the clay fraction in mechanical analysis adopted by the International Society of Soil Science (a settling velocity of less than 1.25 cm. per hour in water at 20° C., nominally a diameter of 2  $\mu$ ). In other words it is sufficient for the majority of investigations to regard the clay fraction of mechanical analysis as the inorganic colloidal material or weathering complex of the soil. Certainly there is no reason to postpone the investigation of this fundamentally important material on the grounds that its preparation requires the use of a supercentrifuge. It happens that many of the earlier investigations, including the ones to be considered here, were made on the so-called "ultra-clay," but there is every reason to believe that the clay fraction obtained by a satisfactory method of mechanical analysis would have given similar results. The exchangeable cations associated partly with this clay and partly with the organic colloids may be determined simply and directly from the total soil, and the results have proved of great value not only in agricultural advisory work but, as shown by K. K. Gedroiz (1929), for some aspects of soil classification. These cations are, however, so reactive and so easily modified by agricultural operations that for a study of the effects of climate and geology they are of less interest than the colloidal anions or clay.

The only systematic study of the colloidal clay of soils from a wide range of climatic and geological conditions is in the admirable work of W. O. Robinson and R. S. Holmes (1924) in the United States Bureau of Soils. Their data consist of full chemical analyses of the colloidal matter separated by dispersing the soil in water, and removing the material coarser than about 0.3  $\mu$  in diameter by settling and by high speed centrifuging. Soil from 30 centres in the United States was selected so as to represent the more important agricultural kinds of soil with a wide range of texture, geographical distribution, and conditions of soil formation, omitting only special soil types such as peats and laterites. At half of the centres samples were taken from two depths, but as these varied only slightly in the chemical properties considered here the deeper samples were omitted from the present analysis.

Robinson and Holmes made a cautious but restricted examination of the relationships between the values for mean annual rainfall and temperature at these centres, and the chemical composition of the 45 clays as exemplified by the molecular ratios  $\text{SiO}_2/\text{Al}_2\text{O}_3 + \text{Fe}_2\text{O}_3$  and  $\text{CaO} + \text{Na}_2\text{O}/\text{Al}_2\text{O}_3 + \text{Fe}_2\text{O}_3$ . They found undoubted but not close inverse relationships between both of these ratios and the rainfall, but they could trace none with the temperature. In general the colloidal matter developed in dry regions was richer in silica, lime, and soda than that in humid regions, but they concluded that there was insufficient evidence to justify the prediction of clay composition from the rainfall or even to show that the mature soils of any region had similar clays. The rainfall effect is to be expected for it is well known that soils of very wet districts, whether podzols in cold ones or laterites in hot ones, have accumulation layers rich in sesquioxides, whilst desert soils generally have highly siliceous clays. This effect is well illustrated in F. J. Martin's (1929) collection of data from a belt across Central Africa. The mean  $\text{SiO}_2/\text{Al}_2\text{O}_3$  values for clay fractions grouped according to rainfall were 1.55, 2.07, 2.39, 3.65 for rainfalls of 10-25, 25-50, 50-80 and over 80 inches respectively.

It is of interest to examine more closely the alleged absence of a temperature effect on Robinson and Holmes' American clays.

#### *Clay Composition and Temperature.*

H. Jenny (1929) has recently re-examined Robinson and Holmes' data in an attempt to show that the more important climatic factors operating in soil formation may be estimated to a first approximation by Meyer's N.S. Quotient (mean annual rainfall in millimetres divided by the difference between the mean saturation pressure and the mean vapour pressure both in millimetres of mercury). He selected from the 30 centres 19 having approximately constant values of this function with the object of securing constant moisture conditions ("Befeuchtung"), and claimed that after rainfall differences are allowed for in this way the molecular ratio  $\text{SiO}_2/\text{Al}_2\text{O}_3$  falls off regularly with increasing temperature. This conclusion has already been quoted with approbation in a recent authoritative work of reference (H. Harrassowitz (1930))—"Jenny wies auch auf ausgezeichneten Zusammenhang des Quotienten  $\text{SiO}_2/\text{Al}_2\text{O}_3$  mit Jahrestemperatur in Nordamerika hin"), and it therefore seems necessary to point out that owing to the small variation in relative humidity in the 19 centres selected by Jenny the denominator of the N.S. Quotient is approximately a linear function of the temperature. Constancy of the N.S. Quotient therefore means that the rainfall increases regularly with

the temperature, and Jenny thus demonstrates nothing more than the rainfall effect originally recognised by Robinson and Holmes in more extensive and representative data. Actually the correlation of the  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios with temperature in Jenny's selection of data is not significant, but if two erratic centres be arbitrarily discarded the negative correlation becomes significant. But as it happens to be accompanied by a much closer correlation between rainfall and temperature ( $r_{RT} = +0.86$  for 17 centres) the whole of the effect must be ascribed to rainfall, as originally concluded by Robinson and Holmes, and not to temperature. It happens, however, that a high positive correlation between rainfall and temperature ( $r_{RT} = +0.57$ ) is also found in Robinson and Holmes' 30 centres and explains their failure to recognise the temperature effect on clay composition.

The association of high values of rainfall and temperature is even more general, for data collected by Jenny from 145 meteorological stations distributed over 45 of the United States still give a highly significant positive correlation coefficient ( $r_{RT} = +0.43$ ). This general relationship not only obscures the effects of rainfall and temperature when tests are made by simple correlations, but brings out clearly one of the essential reasons for the different distributions of soil types in the United States and in Russia. Since evaporation is less at lower temperatures, leaching tends to increase with increase of rainfall and with decrease of temperature. Now in European Russia, where the connection between climatic and soil zones was first recognised, rainfall increases and temperature decreases from south to north. The two factors work in the same direction and leaching increases still more rapidly than rainfall and produces well-defined soil zones across the continent. But in the United States, at any rate in the areas where meteorological stations exist and soils have been studied, rainfall and temperature are positively correlated. Increasing temperature offsets some of the effect of increasing rainfall on drainage and leaching. Comparable leaching conditions may exist at widely different temperatures with consequent differences in native vegetation and in the rates of decomposition of soil organic matter. This not only smooths out soil boundaries and modifies the arrangement of soil zones but makes possible the production of soil types entirely different from those recognised and studied in Russia. It may be mentioned parenthetically in this connection that during the International Soil Excursion in the United States in 1927 one distinguished Russian pedologist on meeting an American prairie soil for the first time refused to accept a statement of the local rainfall on the grounds that it was quite impossible for it to produce such a soil.

*Statistical Examination of Clay Composition and Climatic Data.*

In view of the high correlation of rainfall and temperature in Robinson and Holmes' centres it is evidently desirable to make a fuller statistical examination of their data than has hitherto been attempted and R. A. Fisher's (1930) *Analysis of Variance* is obviously well suited for the purpose. The molecular ratio of  $\text{SiO}_2$  to  $\text{Al}_2\text{O}_3$  (S) was used in preference to the silica-sesquioxide ratio, for, although the two ratios happened to be closely proportional in the soils examined, there is some evidence in other work in this laboratory and elsewhere (*cf.* p. 19) that iron compounds move about in the soil independently of silica and aluminium compounds. The simpler ratio has the additional advantage that it suggests comparison with the alumino-silicates and alumina-silica gels which have been more thoroughly studied than the corresponding iron compounds. For similar reasons the CaO per cent. of the dry colloid (C) was used as a measure of the more mobile bases instead of the ratio  $\text{CaO} + \text{Na}_2\text{O}/\text{Al}_2\text{O}_3 + \text{Fe}_2\text{O}_3$ .

Table I gives these recalculated analytical data and the mean annual rainfall and temperature for all of Robinson and Holmes' soils, omitting the deeper soil where two samples were taken from the same profile. The principles underlying the geological grouping in Table I are discussed later.

The first stage of the analysis is the calculation of the regression coefficients of  $\text{SiO}_2/\text{Al}_2\text{O}_3$  and CaO per cent. respectively on rainfall and temperature. The significance of the regression equation is estimated by evaluating Fisher's statistic  $z$ , which is half the natural logarithm of the ratio of the variance of the values obtained from the regression equation to the remaining variance due to deviations from the equation. For  $\text{SiO}_2/\text{Al}_2\text{O}_3$  the value of  $z$  is well above that likely to arise by chance in 1 per cent. of a series of random samplings ( $P < 0.01$ ); for the CaO the value of  $z$  corresponds to  $P = 0.02$ . Both equations may, therefore, be taken as significant and subject to the standard errors (S.E.) given in Table II.

The decrease of  $\text{SiO}_2/\text{Al}_2\text{O}_3$  with increasing rainfall is thus confirmed but the effect of temperature on clay composition proves to be different from that concluded by either Robinson and Holmes who found no correlation, or Jenny who found a negative one. For constant rainfall clays become more siliceous with *increasing* temperature as would be expected from the effect of temperature on leaching. The relative importance of the rainfall and temperature effects on clay composition may be estimated by evaluating the standard errors of the individual regression coefficients and applying Fisher's  $t$  test. For both

# Soil Clay and Soil Types.

No.	State.	Depth in inches.	Temperature, ° F. T.	SiO <sub>2</sub> /Al <sub>2</sub> O <sub>3</sub> in colloidal clay.		CaO per cent. in colloidal clay.	
				Actual (S).	Calculated from S=S <sub>0</sub> -0.0791 R+0.0699 T	Actual (C).	Calculated from C=C <sub>0</sub> -0.0676 R+0.0644 T
<i>and Melas</i>							
4	Georgia	clay lo.	62	1	2.08	0.31	0.52
19	N. Carolina	clay	64	2	1.99	0.61	0.42
6	Virginia	clay	55	2	1.99	0.17	0.47
21	Maryland	clay	54	2	2.34	0.64	0.69
3	Maryland	clay	53	2	2.17	0.51	0.64
7	Maryland	clay	53	2	1.93	0.93	0.43
<i>B. Limestone</i>							
42	Porto Rico	Ve. Beja clay	77	87	2.00	0.44	0.43
14	Maryland	H. Beja clay	54	34	2.68	1.35	1.14
9	Kentucky	Beja clay	58	72	2.25	0.62	0.75
<i>C. Marine Deposits from Igneous and Metamorphic.</i>							
40	Mississippi	Susquehanna clay	65	1.70	2.26	0.30	0.43
27	N. Carolina	Norfolk fine sandy loam	52	2.08	1.96	0.54	0.26
31	Mississippi	Orangeburg fine sandy loam	63	2.21	2.51	0.51	0.66
33	Maryland	Sassafras silt loam	55	2.32	2.90	0.75	1.03
41	Maryland	Susquehanna clay	53	2.61	2.76	0.24	0.93
20	Mississippi	Lufkin clay	64	4.15	2.66	1.85	0.78

Table I—(continued).

No.	Soil province or region.	Soil type.	State.	Depth in inches.	Rainfall in inches R.	Temperature, °F. T.	SiO <sub>2</sub> /Al <sub>2</sub> O <sub>3</sub> in colloidal clay.		CaO per cent. in colloidal clay.		
							Actual (S).	Calculated from S=S <sub>0</sub> -0.0791 R. +0.0699 T.	Actual (C).	Calculated from C=C <sub>0</sub> -0.0676 R. +0.0544 T.	
D. Marine Calcareous Clay.											
11	III	Crowley silt loam	Louisiana	0-10	55	68	2.91	3.07	0.99	2.51	
16	III	Houston black clay	Texas	0-12	38	65	4.36	4.20	5.01	3.49	
E. Glacial and Loessial.											
12	IV	Dunkirk clay loam	New York	0-8	40	45	2.62	2.49	0.63	0.59	
29	V	Ontario loam	New York	0-12	34	47	2.77	3.10	1.37	1.10	
25	V	Miami silty clay loam	Indiana	0-40	37	50	3.29	3.08	0.92	1.05	
1	V	Carrington loam	Iowa	0-12	32	47	3.37	3.26	1.48	1.24	
23	V	Marshall silt loam	Nebraska	0-14	30	51	3.59	3.70	1.19	1.59	
F. Alluvium from Igneous.											
17	VI	Huntington loam	Maryland	0-8	35	53	2.37	2.61	0.70	0.36	
38	VII	Stockton clay adobe	California	0-38	28	63	3.73	3.79	1.96	1.33	
37	VII	Stockton clay adobe	California	0-12	14	60	3.86	4.76	1.47	2.16	
45	VII	Yolo clay	California	0-18	20	58	4.78	4.14	1.46	1.85	
13	VIII	Fallon loam	Nevada	0-12	5	51	5.41	4.84	2.20	2.28	
G. Alluvium from Loess.											
26	VI	Sharkey clay	Mississippi	0-4	54	66	3.85	3.90	1.41	1.64	
25	VI	Sharkey clay	Mississippi	?	54	66	4.15	3.90	1.92	1.64	
43	VI	Wabash silt loam	Nebraska	0-15	34	52	4.29	4.50	2.19	2.23	

List of soil provinces or regions.

- I Piedmont Plateau Province.  
 II Limestone Valley and Uplands Province.  
 III Atlantic and Gulf Coastal Plains Province.  
 IV Glacial Lake and River Terrace Province.  
 V Glacial and Loessial Province.  
 VI River Flood Plains Province.  
 VII Pacific Coast Region.  
 VIII Great Basin Region.

Table II.—Regression Equations for Clay Composition on Rainfall and Temperature. (Ungrouped data.)

R = Mean annual rainfall in inches.

T = Mean annual temperature in ° F.

S = Molecular ratio of  $\text{SiO}_2$  to  $\text{Al}_2\text{O}_3$  in clay.

C = CaO per cent. in clay.

SE = Standard error.

	Regression equations.		Regression coefficient.	
	SE.		SE.	
$S = 2.063 - 0.0647 R + 0.0626 T$ ..	0.0736	1.304	S on R 0.0124	5.22
			S on T 0.0224	2.80
$C = 0.405 - 0.0441 R + 0.0586 T$ ..	0.836	0.812	C on R 0.0141	3.12
			C on T 0.0254	2.31
(For $P = 0.01$ ) .....		(0.851)		(2.77)

measures of clay composition the negative regression coefficient on rainfall considerably exceeds the value to be expected by chance in 1 per cent. of a series of samplings; the positive regression coefficients on temperature are not so well established but might arise by chance in 1 per cent. for  $\text{SiO}_2/\text{Al}_2\text{O}_3$ , and in 3 per cent. for CaO. Variations of rainfall therefore are more important than those of temperature, but even over the moderate range covered by these data the temperature effect is highly significant. From the ratio of these two regression coefficients it would follow that the  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratio would remain constant when an increase of 1° F. is accompanied by an increase in rainfall of 0.97 inches and that CaO per cent. would be constant for an increase in rainfall of 1.33 inches per ° F. A more detailed consideration of these ratios will be given later.

#### *Relation of Clay Composition to Geology.*

A correlation of clay composition with rainfall and temperature might easily arise from the dependence of climate on topography and geology. The intimate association of climate and geological factors in soil formation has been overlooked almost as frequently as the association of rainfall and temperature already discussed. In the British Isles the complexity of the geological factors influences soil formation and distribution not merely by affording a wide range of rocks for parent material and a highly irregular surface which



prevents a full soil development but also by an indirect action on the climate. Low rainfall areas occur in areas of tertiary and more recent formations ; areas of primary formations generally have high rainfalls and low temperatures. The separation of geological from climatic effects is therefore necessarily difficult but the attempt must be made before the fundamental principles of soil genesis and classification can be placed on a firm and quantitative basis. Robinson and Holmes found the influence of parent material more difficult to estimate than the rainfall effect. They attempted no analysis, but merely illustrated the influence of the age of the soil by referring to the high  $\text{SiO}_2/\text{Fe}_2\text{O}_3 + \text{Al}_2\text{O}_3$  ratios in two soils which are relatively young in the localities where they are now found, and the low values of a group of 10 more mature soils which happen, however, to occur together in a region of relatively constant climatic conditions. They give no other observations on the maturity of the soils as manifested in morphological characteristics and as deduced from topography, and also no information about the geological formations from which the soils were derived. Fortunately the latter omission can be remedied to some extent by using the names of the soil types in conjunction with the information contained in the valuable Bulletin 96 of the U.S. Bureau of Soils. This describes the soils of the United States with the system of classification adopted in 1913. There are 13 main sub-divisions, six soil regions in the Great Plains and the country to the west and seven soil provinces in the east. These are differentiated on general geographical features rather than on soil characters and are consequently inadequate for an attempt to separate climate and geology. Provinces and regions are divided into soil series of the same colour, subsoil, and origin, and these in turn are divided into soil types which have the same texture, colour, structure, subsoil, topography, process of derivation and parent material. Sufficient information is given in Bulletin 96 to justify a classification of the 30 soils sampled by Robinson and Holmes into seven geological groups according to the process of formation of the parent material of the soil. Owing to the small number of samples the grouping is necessarily rough and to restrict the number of groups it is essential to put together such related formations as the various glacial and loess deposits. The geological grouping and the soil regions or provinces are given in Table I and the mean clay compositions within the geological groups are given in Table V, together with certain quantities deduced from the data in Table I.

R. A. Fisher's "Analysis of Variance" provides a ready means of testing (1) the efficiency of this geological grouping ; (2) the significance of the regressions on rainfall and temperature after the effects of this geological grouping

have been eliminated; and (3) the significance of the geological effects after elimination of the average effects due to rainfall and temperature.

Table III.—Analysis of Variance for Geological Grouping.

	Degrees of freedom.	S ( $\text{SiO}_2/\text{Al}_2\text{O}_3$ ).		C (CaO) per cent.	
		Sum of squares.	Standard error.	Sum of squares.	Standard error.
Total .....	29	29.289	—	25.942	—
Between geological groups .....	6	17.891	—	13.179	—
Within geological groups .....	23	11.398	0.704 ( $z=0.897$ )	12.763	0.745 ( $z=0.688$ )
(For $P = 0.01$ , $z = 0.656$ ) ..					

Table III shows that the grouping on a geological basis has proved even more efficient than the regression equations on climatic factors as a means of estimating the clay composition from external conditions (for  $\text{SiO}_2/\text{Al}_2\text{O}_3$  the standard error for the regression on R and T is 0.736 and for the geological grouping 0.704).

#### *Relation of Clay Composition to Geology and Climate.*

At this stage the analysis serves to illustrate an impasse often encountered in soil classification and in other geographical and ecological fields. Here and elsewhere where the properties to be investigated can be expressed quantitatively, the qualitative geological grouping and the regression on climatic factors can be combined in the analysis. The two variances for "between groups" and "within groups" are each further divided into a fraction expressed by average regression equations on rainfall and temperature and a remainder from which a final standard error of the combined analysis is obtained (Table IV). In both measures of clay composition the regression

Table IV.—Regression Equations for Clay Composition within Geological Groups on Rainfall and Temperature.

( $S_0$  and  $C_0$  are constants for each geological group.)

	Regression equation.			Regression coefficients.	
	SE.	z.		SE.	t.
$S = S_0 - 0.0791 R + 0.0699 T$ ...	0.512	1.197	S on R	0.0063	12.52
			S on T	0.0093	7.52
$C = C_0 - 0.0676 R + 0.0544 T$ ...	0.633	0.848	C on R	0.0210	3.22
			C on T	0.0306	1.77
(For $P = 0.01$ ) .....		(0.877)			(2.83)

equations for "between groups" are not significant, i.e., the difference between the geological groups are independent of the mean values of rainfall and temperature for those groups. It follows, therefore, that within the geological groups the regression equations are more efficient than those derived from the original ungrouped data and that the association of clay composition with climatic factors is not a chance effect due to a common dependence on geology. The validity of the average climatic effects is placed on a firmer basis and the clay composition may be estimated from the geological grouping and average regressions on rainfall and temperature with standard error of 0.51 for  $\text{SiO}_2/\text{Al}_2\text{O}_3$  and 0.63 for CaO per cent.

The values of  $\text{SiO}_2/\text{Al}_2\text{O}_3$  and CaO per cent. calculated from these regression equations are compared with the actual values in Table I and the  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios are given graphically in fig. 1 together with the constants  $S_0$  for the geological groups. When it is remembered that no special care had been taken in the collection of soil samples to secure centres in which the soils were fully developed and in stable equilibrium with the climate, it will be seen that the combination of a geological grouping with the simplest climatic factors accounts for a large fraction of the total variation in clay composition. A few of the more erratic soils will be referred to subsequently. The improved fitting obtained by eliminating some of the geological effects improves the accuracy and utility of the regression coefficients on rainfall and temperature, especially for  $\text{SiO}_2/\text{Al}_2\text{O}_3$ , and in the remainder of this paper the regression coefficients in Table IV will be used in preference to those for the ungrouped data in Table II.

The regression equations provide constants for each geological group which are independent of the climatic conditions, viz., the quantities ( $S_0$  and  $C_0$ ) corresponding to conditions in which the negative rainfall effect equals the positive temperature effect. These values are tabulated in Table V in order of increasing values of  $S_0$  and show that, whereas for both the groups of alluvial soils the mean value of  $\text{SiO}_2/\text{Al}_2\text{O}_3$  exceeds 4.0, these high values have been produced by quite different forces. For alluvium from igneous and metamorphic rocks the geological factor is not significantly greater than that for the residual soils from similar rocks; the high value is to be ascribed to weathering in an arid climate. On the other hand the alluvium from loess has the highest geological factor of the series and a relatively low climatic factor. It is clear from these extremes that attempts to relate clay composition to climatic factors must involve a preliminary elimination of geological factors either by some such method as the above or by a careful selection of uniform parent

Table V.—Mean Values of  $\text{SiO}_2/\text{Al}_2\text{O}_3$  and  $\text{CaO}$  per cent. in Clay for Geological Groups.Climatic factor for  $\text{SiO}_2/\text{Al}_2\text{O}_3 = 0.0699 \text{ T} - 0.0791 \text{ R.}$ Climatic factor for  $\text{CaO}$  per cent. =  $0.0544 \text{ T} - 0.0658 \text{ R.}$ 

Geological factor = mean of group — climatic factor.

Geological group.	Igneous and meta-morphic.	Alluvium from igneous.	Lime-stone.	Marine deposits from igneous.	Glacial and loessial.	Marine deposits (calcareous clays).	Alluvium from loess.
Number of soils .....	6	5	3	6	5	2	3
Mean of rainfall in inches .....	44.7	20.4	52.7	49.2	34.6	46.5	47.3
Mean temperature in ° F. ....	56.8	56.8	63.0	58.7	48.0	66.5	61.3
<i><math>\text{SiO}_2/\text{Al}_2\text{O}_3</math> in clay.</i>							
Actual mean, $\text{Sg}$ .....	2.07	4.03	2.31	2.51	3.13	3.64	4.10
Geological factor, $\text{Sg}$ .....	1.63	1.67	2.07	2.30	2.51	2.67	3.56
Climatic factor .....	0.44	2.36	0.24	0.21	0.62	0.97	0.54
Standard error .....	0.21	0.23	0.30	0.21	0.23	0.36	0.30
<i><math>\text{CaO}</math> per cent. in clay.</i>							
Actual mean, $\text{Cg}$ .....	0.53	1.56	0.77	0.68	1.12	3.00	1.84
Geological factor, $\text{Cg}$ .....	0.46	-0.15	0.90	0.81	0.85	2.53	1.70
Climatic factor .....	0.07	1.71	-0.13	-0.13	0.27	0.47	0.14
Standard error .....	0.26	0.28	0.37	0.26	0.28	0.45	0.37

material or alternatively for fully developed soils by a demonstration that the clay composition is independent of geological factors.

*The Ratio of Silica to Alumina in Soil Clay.*

Although the data are insufficient to justify detailed discussion of the individual geological factors the results in Table V suggest that under comparable climatic conditions  $\text{SiO}_2/\text{Al}_2\text{O}_3$  increases steadily with increasing reworking and water transport of the parent material. The lowest values of the geological factors are for soils derived from igneous rocks either directly as sedentary soils or indirectly with one stage of transport for relatively short distances by mountain rivers. Intermediate geological factors are given by the Tertiaries of the Atlantic and Gulf Coastal Plains which are known to have been derived from igneous and metamorphic rocks and also by the glacial and loessial material much of which had undoubtedly been transported in water before and after glaciation. The most siliceous clays are for the alluvium carried by the Mississippi River and its tributaries from the loessial material of the Great Plains. It is not possible to decide from the present data whether the high  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios of the clay fractions of soils derived from relatively recently reworked deposits depend on the greater immaturity of the soils or on the changes in minerals and colloidal material produced by the reworking. Until further information is available on the degree of development of the soil profiles and the thoroughness of the dissection of their physiographical regions, it would appear simpler to summarise the influence of the geological nature of the parent material in the statement that transport and weathering in rivers and coastal waters returns to the clay, or to material weathering to clay in the soil, some of the silica that is removed from it by the processes of weathering in the acid soils of humid regions. In arid regions the clays remain highly siliceous in both mature and young soils presumably because they are formed and remain in the presence of relatively large amounts of soluble silicates in alkaline solutions.

The recognition of two dissimilar sources of highly siliceous clays accounts for some of the unexpected results contained in the earlier clay analyses and for some of the irregularities in the present data. Thus in the earliest British clay analyses, A. D. Hall, and E. J. Russell (1911) found several clays in South-East England with  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios above 4.0. These values are comparable with those obtained later by A. F. Joseph (1924) in the more arid parts of the Sudan. Such similarities in widely different regions appear irreconcilable with important climatic effects until it is realised that many of

Hall and Russell's soils were derived from tertiary and recent formations repeatedly reworked in water. In Scotland and North Wales the high leaching and the great age of the geological formations combine to give low  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios in the clays, though here too there is some indication that glacial drift soils have higher  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios than sedentary ones. G. W. Robinson (1928) in advancing the hypothesis that the primary product of the chemical weathering of mineral silicates is a mixture of kaolinite and nontronite (or of other hydrated silicates with silica-sesquioxides ratio of 2.0) has already suggested that the clay complex of estuarine and other littoral sediments is enriched in silica by the concomitant precipitation of the silicic acid present in river water. He gives for the mean values of the silica-sesquioxide ratio of the clay fractions of North Wales soils 1.90 for those derived from crystalline or consolidated rocks and 2.67 for those derived from alluvium or unconsolidated sediments.

Again, in a few early analyses by W. T. McGeorge (1917) of Hawaii soils the clay fractions from areas of very high rainfall had much lower  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios (0.4, 0.7) than from those with moderate rainfall ( $\text{SiO}_2/\text{Al}_2\text{O}_3 = 1.3, 1.6, 1.7, 1.7, 1.9, 2.4$ ), but it happens that the most siliceous clay ( $\text{SiO}_2/\text{Al}_2\text{O}_3 = 3.0$ ) is from a soil which differs completely from the others and resembles the highly plastic clay adobe soils of California. This soil is found only in gullies and valleys and is clearly derived from transported material weathered in contact with highly siliceous waters. The grosser discrepancies between the actual and calculated values in Robinson and Holmes' data in Table I and fig. 1 may be accounted for in a similar way. In the humid regions the Lufkin clay which has a higher  $\text{SiO}_2/\text{Al}_2\text{O}_3$  than that calculated happens to be from a very raw unweathered subsoil of marine sediment in which the geological effect still masks the climatic one. In the arid region the Stockton clays, one of which has a much lower  $\text{SiO}_2/\text{Al}_2\text{O}_3$  than that calculated, were formed under bad drainage conditions, and have probably been subjected to more leaching than is indicated by their present rainfalls. Allowance for this earlier leaching would make the regression lines fit more closely and yield a geological factor for igneous alluvium appreciably above that for the residual igneous material.

The joint action of geological and climatic factors may alternatively be described in the statement that young soils derived from relatively recent reworked deposits tend to have more siliceous clays than those of old soils derived directly from igneous and metamorphic rocks. It is not, of course, possible to decide from this analysis whether the prime product of weathering

in the soil is a colloidal material with a composition approximating to  $5 \text{ SiO}_2$ ,  $1 \text{ Al}_2\text{O}_3$ , stable in the alkaline conditions of arid regions but breaking down progressively to  $2 \text{ SiO}_2$ ,  $1 \text{ Al}_2\text{O}_3$  with leaching in acid soils, or whether the latter is formed first and becomes more siliceous by contact with relatively concentrated alkaline silicate solutions in arid soils or with dilute silicate solutions in prolonged river transport. The facts that the highly siliceous material is stable in arid regions and the less siliceous one is known to break down still further to laterites and bauxite under intense leaching at high temperatures, point to the more siliceous clay as the primary product of weathering. In any case the present analysis suggests that colloidal clay may be regarded as a mixture of two forms, which may even be present as a core and coating in the same particle, the one determined by the parent geological material and the other by the climatic conditions in which the clay was formed. In young soils the geological and in old soils the climatic effect predominates. The separation of effects due to the age of a soil from those due to the mineral composition of its parent material presents a more complex problem, but one which is capable of treatment by the above methods when a sufficient body of independent data is available.

#### *Variation of Clay Composition in the Soil Profile.*

After the rest of the present paper had been prepared for publication a further communication from the U.S. Bureau of Chemistry and Soils was received in which I. A. Denison (1930) gave the results of a detailed examination of the clay fractions of samples taken on a profile basis from seven soils selected as representing four widely different groups of soils and with different parent materials and degrees of maturity of the soil within one of the groups. Although the number of soils is not sufficient to distinguish between the soil age and mineralogical composition, it is of interest to examine the extent to which the conclusions already drawn facilitate the interpretation of profile data. Table VI gives Denison's data recalculated to  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios and with the horizons of the profiles grouped on a common basis (A is a leached surface layer, B an accumulation layer, and C partially modified parent material. For ease of comparison in one soil the uppermost of three C horizons is treated as a B horizon since no B horizon was analysed, and the description is not inconsistent with this grouping). No climatic data were given, but sufficiently accurate values were obtained from atlases to justify the calculation of approximate climatic factors from the regression equation for  $\text{SiO}_2/\text{Al}_2\text{O}_3$  on

Table VI.—Clay Composition of Profile Horizons (Denison's data).

Number	1	6	4	5	2	3
Soil type	Podsol	Rendzina	Red soil Immature Gneiss	Red soil Partial Granite	Red soil Full Gneiss	Red soil Full Mica schist
Profile development	Glacial drift	Marl	—	+0.1	—0.7	+0.1
Parent material	0.7	0.7	—0.7	—	—	—
Climate factor for $\text{SiO}_2/\text{Al}_2\text{O}_3$	0.7	0.7	—	—	—	—
<i>SiO<sub>2</sub>/Al<sub>2</sub>O<sub>3</sub> in clay fraction.</i>						
Horizon A	3.55	2.95	1.75	1.86	1.82	1.68
" B	3.44	2.85	1.70	(C <sub>1</sub> ) 1.65	1.59	1.39
Upper C	—	—	—	1.02	1.25	1.86
Lower C	5.33	3.10	1.76	0.91	1.20	2.08
<i>Fe<sub>2</sub>O<sub>3</sub>/Al<sub>2</sub>O<sub>3</sub> in clay fraction.</i>						
Horizon A	0.21	0.22	0.19	0.11	0.23	0.22
" B	0.40	0.21	0.14	(C <sub>1</sub> ) 0.07	0.25	0.25
Upper C	—	—	—	0.04	0.17	0.29
Lower C	0.56	0.21	0.14	0.04	0.08	0.15



rainfall and temperature given in Table IV. (The climatic data for the hilly districts of the red soils are the least accurate.)

The A horizons, presumably owing to their greater acidities and organic matter contents, have consistently higher  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios than the B horizons. (Difference 0.14, standard error 0.025.) The  $\text{SiO}_2/\text{Al}_2\text{O}_3$  of the A and B horizons are much less variable than those of the C horizons, especially in the groups of Red Soils. The true soil horizons thus show a closer approach to equilibrium with the climate. There is a significant correlation ( $P = 0.01$ ) between the clays of either the A or the B horizons and the climatic factors, but the B clay is also significantly correlated with the C clay. (The composition of the B clay may be expressed satisfactorily ( $P = 0.01$ ) by an equation of the type

$$\text{SiO}_2/\text{Al}_2\text{O}_3 \text{ of B} = 1.28 + 0.41 (\text{SiO}_2/\text{Al}_2\text{O}_3 \text{ of C}) \\ + 0.53 (\text{Climatic Factor}).$$

The C clays are clearly more influenced by the geological formations than the A or B clays, but even the C clays show some correlation with climate and cannot, therefore, be taken as independent measures of the contribution of the geological factors to the soil composition. In other words the C material is not a primary weathering product independent of climate. This is explained when the age of the soils and maturity of the profiles are considered. Two of the soils are immature. The Rendzina (No. 6) is a young soil in which the development of the profile has been retarded by its heavy texture and high calcium content and one of the red soils (No. 4) was definitely selected on morphological and topographical evidence as being immature. In both of these, the  $\text{SiO}_2/\text{Al}_2\text{O}_3$  of the C clay is similar to that of the A clay which is in fact but slightly altered C material. The three other Red Soils are extremely old, and it would appear that the whole profile has been influenced by soil forming processes with an accumulation of alumina relative to silica even in the horizon of partially disintegrated rock. The influence of the mica in the parent rock is, however, still to be found in the C clay of soil No. 3.

The higher  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios in the C material of the recent reworked sediments is in complete agreement with the conclusions already drawn from Robinson and Holmes' data. The Tschernosem (No. 7) and the Podsol (No. 1) are derived from similar glacial drift materials and in regions of the same temperature; the greater accumulation of the aluminium relative to silica in the A and B clays of the Podsol is in accordance with the higher rainfall and leaching in which they have been formed.

It may be noted also that although the American Bureau of Soils invariably quotes  $\text{SiO}_2/\text{Al}_2\text{O}_3 + \text{Fe}_2\text{O}_3$  ratios in preference to  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios, an examination of the  $\text{Fe}_2\text{O}_3/\text{Al}_2\text{O}_3$  ratios supports the choice of the simpler ratio in this paper. The relative distribution of iron and aluminium within the profile is itself markedly influenced by climate and geology, and it may well prove to be more closely connected with the soil morphology than the ratio of silica to one or both of the sesquioxides.

Here as in other respects, the immature Rendzina shows no sign of segregation of clay constituents. The four red soils from igneous rocks have very low  $\text{Fe}_2\text{O}_3/\text{Al}_2\text{O}_3$  values in the lower C clay, with higher values in the A material in all cases, and still higher values in the B or upper C horizons in the two fully developed soils. There is thus distinct evidence of accumulation of iron relative to aluminium in the course of soil formation at relatively high rainfalls and temperatures. The glacial drift soils on the other hand have high  $\text{Fe}_2\text{O}_3/\text{Al}_2\text{O}_3$  ratios in the C material and progressively lower values in the B and A horizons, especially in the highly leached Podsol. Such indications of a differential effect of climatic and associated vegetational factors emphasise the advantage of considering iron and aluminium separately. The iron is probably influenced to a much greater extent than aluminium by the decomposition of soil organic matter through either reduction or the formation of co-ordination compounds.

#### *The Relative Effects of Rainfall and Temperature on Drainage.*

The examination of the influence of the mode of formation of parent material has demonstrated that the correlations found between  $\text{SiO}_2/\text{Al}_2\text{O}_3$  or CaO per cent. of the clay fraction, and rainfall and temperature do not arise from a chance association of geological and climatic factors within the small number of centres available. On the other hand it has shown that the elimination of these geological effects improves the accuracy of the relationship between clay and climate. The statement that for soils from similar parent materials a rise of  $1^\circ \text{F.}$  requires an additional 0.88 inches of rain to maintain constant  $\text{SiO}_2/\text{Al}_2\text{O}_3$  or of 0.80 inches for constant CaO per cent. in soil clay suggests that these factors measure the average effect of temperature and associated factors on leaching. It therefore becomes of interest to examine more directly the relative effects of rainfall and temperature on the drainage through soil. A sufficient number of lysimeters is not available and the measurements of evaporation made for plant physiological or engineering

purposes from free water surfaces or from various forms of atmometer bear little relation to the water losses from soil which may remain low for long dry periods even in atmospheres of high evaporating powers. Some substitute for a series of lysimeters in different climates may be obtained by examining the mean monthly values at a single centre. The three Rothamsted gauges are well adapted for examining the effect of temperature since the rainfall is fairly uniformly distributed throughout the year. The extreme values of monthly rainfall differ by only 36 per cent. of the mean value, and although there is a relatively dry spell in spring and summer the correlation between mean monthly rainfalls and temperatures is quite negligible. The three gauges are 1/1000 acre in area, 20, 40, and 60 inches deep respectively, and are kept free from vegetation. They were built round undisturbed blocks of a heavy loam on clay, and, as temperature records were not taken until they had been in operation for 7 years, irregularities due to drying and soil movements during their construction are eliminated by taking the mean values for the 51 years with full data. As the three gauges give similar results except for slight systematic differences probably due to irregularities in the distribution of stones and channels within the soil blocks the mean values for the three gauges were used. The data and statistical analysis are given in Table VII. The reduction of drainage by high summer temperatures is so great ( $r_{DT} = -0.77$ ,  $P < 0.01$ ) that it entirely masks the rainfall effect ( $r_{DR} = +0.47$ ,  $P = 0.1$ ). Both partial correlation coefficients ( $r_{DT.R} = -0.97$  and  $r_{DR.T} = +0.94$ ) are, however, highly significant and together account for a variance of 5.080 out of a total of 5.323. The significance of these relationships is shown even more clearly by the  $z$  test of the regression equations and the  $t$  test of the individual regression coefficients given in Table VII. The results become more comparable with those already derived from the examination of American clays if 12 times the mean monthly values of drainage and rainfall be regarded as equivalent to mean annual values at the appropriate temperature. The regression coefficients would then become  $+1.112$  inches of drainage per inch of rain and  $-0.834$  inches of drainage per degree Fahrenheit. To maintain constant drainage a temperature rise of  $1^{\circ}$  F. must be accompanied by 0.75 inches more rain. This agrees closely with the values 0.88 inches and 0.80 inches per  $^{\circ}$  F. required to maintain constant  $\text{SiO}_2/\text{Al}_2\text{O}_3$  and  $\text{CaO}$  per cent. respectively in Robinson and Holmes' 30 clays and serves to confirm the interpretation of the effects on clay of rainfall and temperature in terms of drainage and leaching.

Table VII.—Rothamsted Drain Gauge Data. Mean Monthly Values for Average of 3 gauges (20 inches, 40 inches and 60 inches deep) for 51 years, 1878–1928.

	Rainfall, R.	Temperature, T.	Drainage.	
			Actual, D.	Calculated from R and T.
	inches.	° F.	inches	inches
January . . . . .	2.303	37.5	2.039	1.87
February . . . . .	2.112	38.7	1.686	1.58
March . . . . .	2.072	41.1	1.209	1.37
April . . . . .	2.016	45.5	0.717	1.00
May . . . . .	2.052	51.9	0.539	0.59
June . . . . .	2.253	57.0	0.604	0.46
July . . . . .	2.681	60.6	0.730	0.59
August . . . . .	2.774	59.8	0.796	0.85
September . . . . .	2.322	55.6	0.793	0.64
October . . . . .	3.131	48.6	1.820	2.02
November . . . . .	2.745	42.1	2.105	2.05
December . . . . .	2.866	38.5	2.509	2.43
Mean	2.444	48.1	1.296	1.30

Regression Equations of Drainage on Rainfall and Temperature. (Mean Monthly Values.)

	Regression equations.				Regression coefficients.		
	SE.	z found.	z for P=0.01.		SE.	t found.	t for P=0.01.
$D = -0.821 + 0.866 R$ . . . . .	0.644	0.518	1.333	D on R	0.516	1.68	3.17
$D = 4.282 - 0.0621 T$ . . . . .	0.462	1.603	1.333	D on T	0.016	3.87	3.17
$D = 1.920 + 1.112 R - 0.0695 T$ . . . . .	0.164	2.100	1.154	D on R	0.133	8.37	3.25
				D on T	0.006	12.04	3.25

### *Climatic Factors in Relation to the Distribution of Soil Types.*

The possibility of accounting for considerable fractions of the variation in clay composition and drainage in terms of simple regressions on annual rainfall and temperature suggests the desirability of examining the consistency of these and other associated climatic factors for regions known to have similar soil types, and of attempting to deduce quantitatively the interaction of rainfall and temperature on soil formation from the actual distribution of soils. There

has been general agreement that neither rainfall nor temperature alone is sufficient to characterise the soil forming action of the climate and several attempts have been made to allow for the variation of evaporation with temperature. Most interest had been aroused by Lang's "Regenfaktor" (rainfall in centimetres  $\div$  temperature in degrees C.) and Meyer's "N.S. Quotient" (rainfall in centimetres  $\div$  absolute saturation deficit in millimetres Hg). Both are open to obvious objections especially for cold regions where the factor is necessarily high. The evidence advanced to support them has generally been limited to data from a few isolated centres and to the demonstration that maps of the distribution of the function over Europe and U.S.A. bear some superficial resemblance to maps of the broadest soil divisions. Recently Jenny (1930) has attempted a more exact treatment. He tabulated the relevant data from 65 meteorological stations in U.S.A. and grouped them into eight soil regions from soil maps prepared by C. F. Marbut (1928), the Head of the United States Soil Survey. Jenny claimed that the more complex function of Meyer is more accurate than that of Lang when judged by the range between the extreme values found within each group of soils. As this criterion is inadequate these functions and certain related values were examined by applying the  $z$  test to Jenny's data. With an efficient climatic function the variance between the soil groups should be high in comparison with the variance within the soil groups. (To avoid over-emphasising the group of yellow and red soils which contained twice as many soils as any other group in Jenny's collection of data the 19 centres were reduced to 10 by discarding alternate centres when arranged in order of increasing temperature.)

The values of  $z$  given in Table VIII show that although Meyer's "N.S. Quotient" is slightly better than Lang's "Regenfaktor," neither has any advantage over the rainfall or temperature alone as a means of grouping the

Table VIII.—Efficiency of Grouping of Climatic Factors by Grouping Centres according to Soil Properties.

(56 centres divided into 8 soil groups, Jenny's collection of data based on Marbut's maps.)

$z$	Climatic Factor.
2.28	Vapour Pressure in millimetres (V.P.) — calc. from T. and R.H.
2.17	Rainfall in centimetres (R).
2.12	Saturation Vapour Pressure in millimetres Hg (P) — calc. from T.
2.04	Temperature in ° C. (T).
1.92	Meyer's "N.S. Quotient" $[R/P(1 - RH)]$ .
1.73	Lang's "Regenfaktor" $(R/T)$ .
1.63	Relative Humidity (RH).
1.59	Saturation Deficit $[P(1 - RH)]$ .

climatic values in accordance with the soils. Further the arrangements of the soil groups in order of climatic values is unsatisfactory. Although Lang's and Meyer's functions agree in assigning the extremes of climates to grey desert soils and podzols, they place the climate of the prairie soils with those of the highly leached red soils, yellow soils and iron laterites; Lang's factor assigns an even wetter climate to the Northern Tschernosem which Marbut has classed among the Pedocals or soils of arid climates. Such failures destroy the value of these combined climatic functions.

The relative humidity which has been proposed in Russia (Kaminski, 1924) as an improvement on these two factors, arranges the soil types roughly in the order expected from their compositions, but the  $z$  test shows that it is inefficient for grouping. The mean vapour pressure has proved the most effective single criterion for separating groups, but the arrangement is naturally the same as for temperature and is not in accordance with the soil composition. The mean vapour pressure has the advantage over Meyer's factor that it represents a simple physical quantity whereas the N.S. Quotient has meaningless dimensions, but it is rarely determined directly and its indirect calculation requires data not generally available. The mean annual rainfall thus remains the most useful single criterion for climatic grouping and mean annual temperature is almost as efficient.

An examination of the relationship of rainfall to temperature within the soil groups shows the reasons for the failure of the combined climatic functions. When the means of the groups are arranged in order of increasing rainfall the regression coefficients of rainfall on temperature within the groups increases from insignificant values in the drier regions to high positive values for the Prairie Soil and Podsol regions, and falls again to low values in the very humid regions of Red Soils and Ferruginous Laterites. This regression coefficient may be expressed as a quadratic function of rainfall. Some such relationship is to be expected, for although no moderate variation in temperature could alter the essential processes of soil formation in regions of very low or very high rainfall, it would, however, greatly influence the leaching in areas of moderate rainfall. The coefficients will not, however, be presented and discussed here as they are not derived from sufficiently representative data. Jenny omitted not only the most widely distributed group, the Brown Forest soils, but also left out those parts of the Prairie belt in which the N.S. Quotients and other climatic data deviated widely from the mean. But even the rectification of these points still provides a poor sample of the climates of the principal soil regions. The necessity of using relative humidities to calculate

the N.S. Quotient restricts the choice of centres to first-class meteorological stations among which an undue proportion are either in large cities or on the coast. Until much further progress has been made in the correlation of soils and climate by rigid analysis, it seems advisable to concentrate attention on the simplest climatic data whose distribution is sufficiently well-known to be mapped with an accuracy greatly exceeding that of the soils themselves, and to postpone to a later stage the use of more complex functions involving either other factors or seasonal distributions.

The annual rainfall and temperature were therefore examined on a more representative basis by collecting data from large scale maps at regular intervals of 2° of latitude and longitude. Temperatures were corrected for altitude by means of the factor used in preparing the map of sea-level temperatures. These soil groups were obtained from photographic enlargements of Marbut's maps, but the selection of centres was restricted to the nine soil groups which have been sufficiently studied to have received distinctive names. This gave an area of approaching two million square miles and included the eastern half of U.S.A. and a band across the middle of the western half. The mean values of rainfall and temperature with their standard errors and the regression coefficients of rainfall on temperature within the nine soil groups are given in Table IX in order of increasing rainfall.

Marbut's system of soil classification and maps are well adapted for an examination of the nature of the association of climate and soil properties, for they are based wholly on the features of the soil and not, as in some European systems, on assumed relationships of soil to climate.

The two broadest soil divisions *Pedocals*, in which calcium carbonate, and *Pedalfers*, in which sesquioxides have accumulated, are seen to occur in semi-arid and humid regions respectively. It is obvious too from the standard errors of the mean rainfalls and temperatures that the group of Prairie Soil differs from all others in extending over a very wide temperature range (actually from 3° to 22° C.). There is considerable variation in the relationship of rainfall to temperature within the soil groups. Except for the insignificant values for Grey Desert Soils and Brown Semidesert Soils the correlation is positive and reaches a highly significant value in the large group of Brown Forest Soils. Strangely enough this group was omitted by Jenny from the data previously considered.

In the second part of Table IX the efficiency of the association of the climatic data and soil properties is examined by applying the *z* test to (a) the 9 groups, (b) a class of Marbut's 4 *Pedocals*, (c) a class of Marbut's 5 *Pedalfers*, and (d)

Table IX.—Rainfall and Temperature of Regions of Established Soil Types (Marbut's Soil Classification and Maps).

## (a) Individual Soil Groups.

Soil group.	Number of centres.	Mean values rainfall (R in cm.).	Temperature (T in °C.).	Standard errors of means.		Regression of R on T within groups.	P for equal regression by chance.	R - 3.3 T (leaching factor).
				R.	T.			
<i>Mid-latitude Temperate Pedocals.</i>								
IV-4 Grey .....	13	25	8.5	6.5	3.0	-1.4	>0.1	-3
IV-3 Brown .....	6	33	5.4	6.8	2.5	-2.9	>0.1	15
IV-2 Chestnut coloured .....	5	42	8.3	5.0	1.8	2.3	0.06	14
IV-1 Tchernosem .....	5	60	11.0	11.0	2.4	3.8	0.08	23
<i>Pedalfera.</i>								
V-6 Prairie soils .....	20	87	11.5	13.6	4.8	1.2	0.03	49
V-2 Podzols .....	12	87	5.1	13.3	1.4	5.5	0.05	70
V-3 Brown forest soils .....	32	104	10.1	20.9	2.8	5.2	<0.01	70
V-4 & 5 Red and yellow soils .....	18	127	17.1	13.4	1.8	1.7	>0.1	70
V-8 Ferruginous laterites .....	6	138	20.7	10.7	1.7	2.0	>0.1	69

## (b) Classes of Soil Groups.

	Number of centres.	Efficiency of grouping.			Significant regression coefficients of R on T (for cases in which $P < 0.01$ ).		
		z for rainfall.	z for temperature.	z for $P = 0.01$ .	All centres.	Within groups.	Between groups.
(a) All groups .....	117	2.24	1.74	0.52	4.64	2.36	—
(b) Mid-latitude Pedocal groups .....	29	1.70	1.11	0.77	2.73	—	—
(c) Pedalfer groups .....	88	1.59	1.82	0.64	3.09	—	—
(d) Pedalfer groups (omitting Prairie soils) .....	68	1.50	2.31	0.71	3.54	4.48	3.34



a class of highly leached acid soils obtained by removing the Prairie Soils from the Pedalfer class. In all four classes both rainfall and temperature are significantly associated with the soil groups. As would be expected when all 9 groups are considered together the soils are related more closely to rainfall than that to temperature, i.e., rainfall gives a higher  $z$  than temperature. After eliminating the broad division into semi-arid and humid regions the rainfall remains of greater importance than temperature for the class of Pedocals. Temperature on the other hand becomes the dominant factor (i.e., has the higher  $z$ ) for the Pedalfers, especially when the Prairie Soils group is omitted. It is clear, therefore, that no single combined function of rainfall and temperature can serve as an effective measure of climate over the whole range of soils represented within even the temperate climatic zone and failure of such functions as Lang's and Meyer's is inevitable. The importance of rainfall in the semi-arid region is obvious and the dominance of temperature in the humid belt is doubtless to be explained partly through an effect on evaporation and partly through its influence in determining the type of natural vegetation maintained at any rainfall level. The abnormality of the prairie belt is again shown in the low but significant regression coefficient of rainfall on temperature. This band may justly be regarded as transitional between the humid and the semi-arid regions and doubt may be expressed as to the uniformity of its soils. For its greater part rainfall and temperature are closely related and leaching from their joint action is only slightly below that in the belt of Brown Forest Soils, but in Texas and Oklahoma the temperature rises rapidly without a corresponding increase in rainfall. Geographically the Prairie Soil belt is unique, having common boundaries with four of the eight soil groups listed above and with two broad groups, not yet named by Marbut, lying to the north and south respectively of the Tschernosem belt.

The table of significant regression coefficients illustrates once more the general correlation of rainfall and temperature throughout the greater part of the cultivated area of U.S.A. The division of this regression "between groups" and "within groups" for the four broad classes shows that a significant value "within groups" is obtained only for the class of highly leached soils. It also happens that the mean values of the groups within this class show a highly significant regression of rainfall on temperature which is illustrated in the first part of Table IX by the striking constancy of the values of  $(R - 3.3 T^{\circ} C.)$  for the last four soil groups.

A comparison in Table X of the regression coefficients found for highly leached soils in Table IX with those derived earlier in the paper for the production of constant clays from similar parent materials and for constant

Table X.—Relation of Rainfall to Temperature for Constant Soil Formation.

	Centimetres per ° C.
(1) For constant $\text{SiO}_2/\text{Al}_2\text{O}_3$ in clay .....	4.04
(2) For constant CaO per cent. in clay .....	3.78
(3) For constant drainage in Rothamsted lysimeters .....	3.40
(4) From the distribution of groups of highly-leached soils—	
(a) Means of groups .....	3.34
(b) Within groups .....	4.48

drainage in lysimeters shows fair agreement, suggests that the highly leached groups of soils in the Eastern United States have similar clays. These appear to belong to the halloysite ( $2 \text{SiO}_2, 1 \text{Al}_2\text{O}_3$ ) type but may contain some excess of sesquioxides. Contrary to expectations it does not seem that the distinction between the principal soil groups in this highly leached class can be made in terms of the alumino-silicates of the clay fraction. It appears rather that the morphological properties used to characterise them depend more on the distribution of iron and organic matter, and that the determining climatic factor is not leaching but temperature. At high temperatures weathering of minerals and decomposition of organic matter proceed rapidly giving deep soils in which iron is precipitated near the surface (Ferruginous Laterites and Red Soils). At low temperatures the iron and organic matter soils are more stable and are leached down to distinct accumulation horizons (Podsol). The Brown Forest soils represent an intermediate class in which movement of colloidal material is impeded by the higher salt and exchangeable base contents maintained by deciduous broad leaved forest covers.

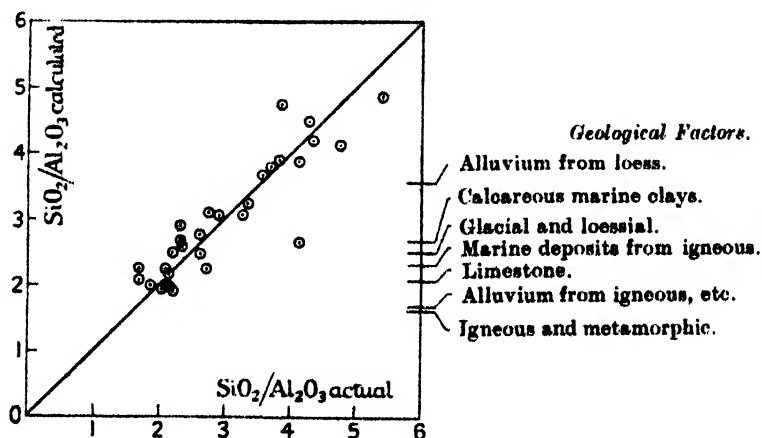
If the factor  $R \text{ cm.} - 3.3 T^\circ \text{C.}$  is used as a rough index of drainage and leaching, the values of the rest of the soil groups in Table IX bring out the intermediate position of the Prairies, and low leaching of the semi-arid regions and the absence of drainage in the Grey Desert Soils. The climates of the greater part of U.S.A. may in fact be classified in relation to soil formation by using such a leaching factor in conjunction with the predominance of temperature in the highly leached and of rainfall in the slightly leached regions. As a first approximation the leaching factor may be taken in round numbers as  $(R - 10/3 T^\circ \text{C.}) \text{ cm.}$  or  $(R + 24 - \frac{3}{4} T^\circ \text{F.}) \text{ inches.}$

#### *Classification of Climates in Relation to Soil Formation.*

(1) <i>High Leaching</i> .....	Podsol	With
(leaching factor about 70 cm.)	Brown Forest Soils	increase
	Red and Yellow Soils	of
	Ferruginous Laterites	tempera-
		ture.

- |   |                         |          |
|---|-------------------------|----------|
| (2) <i>Transitional</i> .....   | Prairie Soils.          |          |
| (leaching factor considerably less than 70 cm., rainfall greater than 70 cm.) |                         |          |
| (3) <i>Low Leaching</i> .....   | Tschernosem             | With     |
| (leaching factor and rainfall both less than 70 cm.)                          | Chestnut Coloured Soils | decrease |
|   | Brown Semi-desert Soils | of       |
|   | Grey Desert Soils       | rainfall |

There is some evidence that greater accuracy would be obtained by using a higher regression coefficient of rainfall on temperature for the leaching factor for Podsolised Forest Soils and Brown Forest Soils and a lower factor for Prairie, Tschernosem and Chestnut Coloured Soils. Such expressions would be in harmony with the fact that the forest groups have a greater, and the steppe and prairie groups a smaller, cover of natural vegetation than the average of the centres used in the statistical analysis of both clay composition and the distribution of soil types in this paper. The data available are too scanty to justify closer analysis at the present stage, but the results have demonstrated the desirability of stating at least the simpler climatic data (mean annual



rainfall and temperature) for all centres where fundamental quantitative data on soils are collected. This is especially desirable in the tropics for the simple relationships discussed here appear to break down at higher temperatures and in any case the Ferruginous Laterites of Florida are widely different from the true Laterites. The collection of exact quantitative climatic and soil data with some geological information is particularly necessary in isolated tropical

countries where soil work has only recently been undertaken in order to provide material for a rigid examination of soil forming processes. It should then become possible to develop sounder systems of soil classification than was possible in Europe where too great a concentration on restricted regions, of the use of inadequately established generalisations on the relationships of climate to soils, led to an excessive use of the parent material or the climatic zone respectively, and impeded the discovery of the more fundamental principles in soil formation.

*Summary.*

(1) An attempt is made to separate the effects on soil formation of two quantitative climatic factors and a qualitative geological grouping by means of a statistical analysis capable of application to other geographical and ecological problems. The data discussed consist of chemical analyses of the clay fractions of 30 representative soils and maps of the distribution of the more important soil groups in the United States.

(2) The existence of a high positive correlation between annual rainfall and mean annual temperature in the agricultural areas of the United States accounts for earlier failures to recognise the influence of temperature on clay composition, and also for the essential differences between the nature and distribution of soil types in the United States and in Russia.

(3) The ratio of silica to alumina in the clay fraction is correlated negatively with rainfall and positively with temperature, and the relative effects of rainfall and temperature on clay are closely parallel to their effects on the amounts of drainage through soil in the Rothamsted lysimeter experiments. This suggests that the amount of leaching is the dominant factor in clay formation.

(4) For comparable climatic conditions the lowest  $\text{SiO}_2/\text{Al}_2\text{O}_3$  ratios in the clay fraction are in sedentary soils from igneous rocks, and the highest in the young soils from sediments repeatedly subjected to reworking in water. Highly siliceous clay may be formed in two ways; by weathering in the presence of soluble silicates in the alkaline soils of arid regions or in the immature soils of humid regions where the parent material has been exposed for long periods to the dissolved silica of river waters.

(5) In the regions of highly leached soils in the United States the relationship of rainfall to temperature corresponds to conditions of approximately uniform drainage and clay composition. Within this belt the individual soil groups are more accurately characterised by temperature than by rainfall or leaching. The group of Prairie Soils occurs in a transitional belt between these highly

leached soils and those of semi-arid regions in which rainfall is the dominant climatic factor in the determination of soil type.

## REFERENCES.

- I. A. Denison (1930), 'J. Agric. Research,' vol. 40, p. 469.  
 R. A. Fisher (1930), "Statistical Methods for Research Workers," 3rd ed.  
 K. K. Gedroiz (1929), 'Kolloidchem. Beihefte,' vol. 29, p. 149.  
 A. D. Hall and E. J. Russell (1911), 'Journ. Agric. Sci.,' vol. 4, p. 182.  
 H. Harrassowitz (1930), 'Blanck's Handbuch der Bodenlehre,' vol. 3, p. 422.  
 E. W. Hilgard (1905), "Soils."  
 H. Jenny (1929), "Soil Research," vol. 1, p. 140.  
 A. F. Joseph (1924), 'J. Agric. Sci.,' vol. 14, p. 490.  
 A. A. Kaminski (1924), quoted by S. S. Neustruev (1927) 'Russian Pedological Investigations,' Leningrad No. 3.  
 C. F. Marbut (1928), 'Proc. First Intern. Congress of Soil Science,' vol. 4, p. 1.  
 F. J. Martin (1929), 'Conference of Empire Meteorologists,' vol. 2, p. 42.  
 W. T. McGeorge (1917), 'Hawaii Agric. Expt. Sta. Bull.,' No. 42.  
 W. O. Robinson and R. S. Holmes (1924), 'U.S. Dept. Agric. Bull.,' No. 1311.  
 G. W. Robinson (1928), 'Nature,' vol. 121, p. 903.

612.621 : 612.826.3

*The Relation between the Anterior Pituitary Body and the Gonads.—  
 Part I. The factors concerned in the formation of the corpus  
 luteum.*

By MARGARET HILL and A. S. PARKES (Beit Memorial Research Fellow,  
 University College, London.)

(Communicated by C. L. EVANS, F.R.S.—Received May 31, 1930.)

## [PLATE 1.]

## I. Introduction.

Ovulation in the normal rabbit is dependent upon copulation (Heape, 1905). Since the same correlation is found with grafted ovarian tissue, as shown by Friedman (1929a) and previously found by Asdell (1926), the stimulus (presumably nervous in nature) set up by coitus does not act directly upon the ovary. In view of the part which the anterior pituitary body is now known to play in controlling ovulation (Zondek and Aschheim, 1927, Smith and Engle, 1927) it is natural to suppose that copulation stimulates the anterior pituitary,

which in turn reacts upon the ovary to cause ovulation. It has, in fact, been shown that hypophysectomy of the rabbit within 1 hour after copulation inhibits the ovulation which would normally occur 10-12 hours later (Fee and Parkes, 1929).

Furthermore, Bellerby (1929) was able to induce ovulation in the unmated oestrous rabbit by the injection of acid extracts of anterior pituitary tissue. Later, Friedman (1929b) obtained the same result with extracts prepared from urine of pregnant women. Both of these authors were able to obtain the effect by one injection, and the influence of this one administration of hormone must have passed off by the time of ovulation. Neither author, however, appears to have studied the subsequent history of the follicle caused to ovulate without the preceding influence of copulation and free from the extraneous stimulus of further injection. It is evident that such a study would indicate something of the factors concerned in initiating the stimulus (presumably hypophyseal in origin) responsible for the luteinisation of the ruptured follicle.

The experiments described in the present paper were concerned primarily with testing the ovulation-producing power of various extracts, but advantage was also taken of the opportunity to ascertain whether a corpus luteum develops automatically after ovulation—and therefore after induced ovulation—or whether some second stimulus, normally dependent upon copulation, is required.

## II. *Material and Technique.*

The rabbits were kept alone for 2-3 weeks before use, and laparotomy was usually performed immediately before injection to ascertain whether ripe follicles were present. Only animals obviously in oestrus were used. The animals described here received a single injection into an ear vein immediately after the first laparotomy. Laparotomy was again performed in 15-18 hours to ascertain whether ovulation had taken place. Some animals were killed at this stage, while others were left for graduated periods up till 30 days after injection. In animals intended for survival the use of urethane or similar anaesthetic was avoided, ether and atropine being used for each laparotomy. To reduce the amount of manipulation no attempt was made to arrive at the exact time of ovulation.

At the time of autopsy selected portions of the ovaries (ruptured follicles, etc.) and part of the uterus were fixed for histological examination, while the mammary tissue was removed and examined as described by Hammond (1925).

### III. *Preparation of Extracts.*

The extracts used were mostly prepared from urine of pregnant women, but a few experiments were carried out with extracts of anterior pituitary tissue and rabbit placenta. The tissue extracts were made by the alkali treatment originally used by Evans (1924) and subsequently by many workers (Parkes, 1929, Teel, 1926). The preparation of the active substance from urine was carried out by modifications of the method used by Zondek and Aschheim (1928) and Evans and Simpson (1929).

*Separation of Œstrin in Urine Extracts.*—Extracts are commonly tested for gonad-stimulating properties on immature mice, and, further, stimulation of the immature ovary is often diagnosed merely by the vaginal reaction. Since this reaction can be caused directly by the injection of Œstrin, which is present in large amounts in urine of pregnancy, such a technique is only valid when the extract to be tested is completely Œstrin-free. In the diagnosis of pregnancy by testing the urine for the gonad-stimulating principle it is essential that either the ovaries are also examined, or that Œstrin is completely eliminated. Even when examination of the ovary is also carried out, it is clearly desirable that the action of the extracts shall not be complicated by the presence of Œstrin. To remove the ovarian hormone Zondek and Aschheim carried out a preliminary ether extraction of the urine after slight acidification. In using this process on our earlier preparations it was found that even five extractions with large volumes of ether were quite inadequate to remove all the Œstrin—our final preparations of the aqueous residue had a strong Œstrus-producing activity in ovariectomised mice. This result is explained by Marrian's (1929) observation that ether is quite ineffective in extracting Œstrin from neutral or nearly neutral solutions, and that strong acidification of the solution is necessary to effect quantitative removal of Œstrin by ether. From the point of view of preparing the gonad-stimulating principle, acidification is undesirable on account of the large amount of salt of neutralisation formed subsequently. In our later experiments, therefore, repeated precipitations with alcohol and acetone were relied upon to remove the Œstrin content. This process gives Œstrin-free extracts, but it is unsatisfactory from the standpoint of preparing Œstrin. It is not easy at the moment to see an ideal method of preparing both Œstrin and the anterior pituitary substance from large volumes of urine.

*Treatment of Urine.*—Various methods were used to remove as much as possible of the inert contents of the urine. The following system was finally adopted. The urine, usually 5 to 10 litres at a time, was evaporated down in

*vacuo* below 45° C. to a small volume and washed out of the flask with a minimum amount of water. The turbid fluid was then centrifuged (filtering being difficult or impossible) until the supernatant layer became clear. The precipitate was re-extracted with enough water to bring the total aqueous extract to one-tenth of the original volume of urine. After centrifuging again, the supernatant fluids were combined and four times the amount of absolute alcohol added, bringing the concentration of alcohol to 80 per cent., and the total volume to one-half that of the original urine. The precipitate was allowed to settle and could then be filtered off on a Buchner funnel. The precipitate was dissolved in water and alcohol added to make up to 80 per cent., the total volume of fluid used in this second extraction being equal to one-fifth the volume of original urine. The material soluble in the 80 per cent. alcohol was highly toxic when administered subcutaneously and possessed little or no gonad-stimulating activity. The precipitate at this stage varied a great deal in nature, being usually a powder, but sometimes a sticky mass difficult to handle. In some of the later preparations, rectified spirit (96 per cent.) was used instead of absolute alcohol to bring the concentration to 80 per cent. alcohol. The oestrin, being readily soluble in 80 per cent., was largely removed at this stage. The precipitate was dried and finally made oestrin-free by successive extraction with absolute alcohol and acetone, in amounts equal to one-tenth the original volume of urine.

The product at this stage was invariably a dry, white powder, easy to manipulate and reasonably free from urea owing to the alcohol extractions. Aqueous solutions, however, tended to be toxic, and it was found that a chloroform extraction materially decreased the toxicity, and was therefore desirable at this stage.\* The yield of powder after chloroform extraction varied from 2-6 gms. per litre of urine, but the majority of batches yielded about 5 gms. per litre. This variation is not surprising in view of the great difference in the nature of the original urine.

*Preparation of Extracts for Injection.*—In some preparations the powder was extracted with successive small amounts of water (roughly totalling 200 c.c. per litre of urine) the extracts being finally combined, evaporated down to a volume representing 30 c.c. per litre of original urine, and used for injection. The method of preparing the final concentration according to the volume of urine used is, of course, unsatisfactory in so far as the concentration of the

\* As regards intravenous injection to rabbits, toxicity depended to some extent on rate of administration. Slow injection—10 c.c. over, say, 30 minutes—caused a far greater toleration of the extract than more rapid administration.



original urine varied very much, but the other possible system, using the amount of powder as a basis, had equal disadvantages.

In other preparations, fractional extraction with ethyl alcohol was resorted to. The powder was extracted with successive small amounts of 50 per cent. alcohol, the alcohol being distilled from the combined extracts and the preparation (50 per cent. alcohol soluble) made up with distilled water to a volume equal to 30 c.c. per litre of urine. Small amounts of 30 per cent. alcohol were then used and this fraction (30 per cent alcohol soluble) prepared for injection in the same way. The 50 per cent. and 30 per cent. alcohol insoluble fraction was found not to contain any detectable activity and was discarded. The 50 per cent. and 30 per cent. alcohol soluble material, however, was highly active when tested on immature mice, rabbits and ferrets. Many of the extracts were tested in graduated dilutions on immature mice, and the results indicated that some 200-300 units per cubic centimetre were present in the total water-soluble extract. The alcohol fractions (50 per cent. and 30 per cent.) possessed less activity, the 30 per cent. having the least. In each case the weight of the unit was of the order of 1 mgm. A complete report on the effects of these extracts on immature mice is being prepared.

No attempts were made to render the extracts isotonic before intravenous injection.

#### IV. *Induction of Ovulation without Copulation.*

Table I summarises the 30 experiments performed on the induction of ovulation without copulation.

Table I. Induction of Ovulation by various Extracts.

Extract used.	Number of animals.	Number dying before ovulation time.	Number ovulated.	Number failing to ovulate.
Ant. pit. tissue (alk.)	2	2	0	0
Urine. Total H <sub>2</sub> O sol.	8	2	5	1
Urine. 50 per cent. alc. sol.	14	1	4	9
Urine. 30 per cent. alc. sol.	5	1	2	2
Rabbit placenta	1	0	1	0
Total	30	6	12	12

The alkali extracts of ox anterior pituitary tissue proved to be highly toxic when given intravenously, both animals used dying before ovulation could have occurred. Of the animals receiving urine fractions, about one-half

ovulated. The amounts given to those ovulating are shown in Table II. Judging from Table I the total H<sub>2</sub>O soluble preparation would appear to be more effective in producing ovulation in the rabbit than either of the alcohol-soluble fractions, but, as shown in Table II, the 50 per cent. alcohol extracts were given in smaller amounts, and there is probably little difference in activity between the two. There appears to be no correlation between the amount

Table II.—History of Animals in which Ovulation was Induced by Injection.

Number of animal.	Nature of injection.		Time of autopsy.	State of		
	Extract.	Amount.		Ovary.	Uterus.	Mammary glands.
		c.c.	hours			
APF5	H <sub>2</sub> O sol. ....	15	15.0	4 r.f.	—	—
APF2 Rt.	" .....	10	15.30	†2 r.f.	—	—
APF18	50 per cent. alc. sol.	5	17.00	5 r.f.	—	—
APF28	Placental .....	10	17.45	5 r.f.	—	—
APF8 Lt.	H <sub>2</sub> O sol. ....	8	18.00	†3 r.f.	—	—
APF4 Lt.	" .....	10	24.30	†2 r.f.	—	—
			days			
APF4	" .....	10	6	5 c.l.	early p.p.	early p.p.
APF2	" .....	10	8	3 c.l.	p.p.	p.p.
				4 b.f.		
APF20	50 per cent. alc. sol.	5	10	9 c.l.	p.p.	p.p.
APF19	" .....	5	14	o.c.l.	un-	p.p.
					developed	
APF23	" .....	5	14	12 c.l.	p.p.	p.p.
APF10	30 per cent. alc. sol.	10	20	o.c.l.	old p.p.	old p.p.
APF9	" .....	10	24	o.c.l.	old p.p.	—
				m.f.		
APF8	H <sub>2</sub> O sol. ....	8	30	"	oestrus	—
APF7	" .....	5	30	"	old p.p.	—

(r.f. = ruptured follicle; c.l. = corpus luteum; b.f. = blood follicle old corpus luteum; p.p. = pseudo-pregnant; m.f. = mature follicle.)

† Ovary obtained by operation.

given and the number of follicles ovulating. In fact, double the dose (of the same extract) effective in one animal may be ineffective in another. In the same way, though the exact time of ovulation was not observed, no great variation occurred according to the dosage given. It may be noted that the smallest effective amount of any extract (5 c.c.) represents rather more than 160 c.c. of original urine.

#### V. Development and Function of the Corpus Luteum after Induced Ovulation.

The formation, development and retrogression of the corpus luteum after induced ovulation appears to be exactly similar to that found after sterile copulation in the normal animal. Further, the correlated growth and subse-

quent atrophy of the uterus and mammary glands are the same as during the period of ordinary pseudo-pregnancy, described by Ancel and Bouin (1929*a*, *b*) and Hammond and Marshall (1914, 1925). This indicates that the corpora lutea formed after induced ovulation perform their usual endocrine functions for the usual time. Table II shows the stages obtained after induced ovulation, together with the data relating to the type of extract used, the number of follicles ovulating and the state of the accessory organs.

Plate 1 shows the state of the corpora lutea and accessory organs at various stages after induced ovulation. The ovary obtained at 6 days after injection contained several healthy nearly solid corpora lutea, while the uterus and mammary glands exhibited the typical signs of early pseudo-pregnancy. At 8 days after injection the development was proceeding. At 10 days the corpora lutea had attained an average diameter of about 3 mm., while the mammary gland and uterus were almost fully pseudo-pregnant. Of the two animals killed at 14 days after injection, one proved to be the only exception in the series. Although the animal was known to have ovulated the corpora lutea were small and the uterus was undeveloped. The mammary glands, however, were well developed. The second animal killed at 14 days showed all the characteristic signs of pseudo-pregnancy. The next stage obtained at 20 days after ovulation showed the regression of pseudo-pregnancy. The corpora lutea were shrinking, having a diameter of about 2.0 mm., and the accessory organs presented the appearance characteristic of the post-pseudo-pregnant period. The later stages obtained showed the gradual return to the oestrous condition. The two animals killed at 30 days had ripe follicles among the old corpora lutea. APF8 was mated on the 29th day and ruptured follicles were found in the ovary on autopsy 20 hours later.

The fact that corpora lutea develop and function after induced ovulation without extraneous assistance shows fairly definitely that the stimulus to luteinisation of the ruptured follicle is set in motion by the act of ovulation, and not as a delayed effect of copulation. In other words, the nervous reflex (probably originating in the vulva or vagina) set up by copulation, and necessary to initiate the ovulation-producing stimulus, is not connected with the subsequent release from the anterior pituitary body of the stimulus to luteinisation. This work therefore extends the somewhat contradictory experiments of Ancel and Bouin (1909*a*), O'Donoghue (1913) and Hammond and Marshall (1925), who have shown that a corpus luteum may sometimes develop after the mechanical rupture of the ripe follicle in the rabbit.

VI. *Results of Failure to Induce Ovulation.*

The subsequent histories were followed of four animals in which injection had failed to induce ovulation. The animals were killed at 2-10 days after injection and in each case numbers of large blood follicles were present in the ovary; in one animal the blood follicles were as much as 4 mm. in diameter. These animals all had normal ripe follicles when injected, and in two cases the extract given had been effective in another animal in the same amount. It is not easy to see why the ripe follicle if stimulated, but insufficiently so to ovulate, should experience the extraordinary hæmorrhage leading to the formation of these large blood follicles. Such follicles are, however, not uncommon in untreated rabbits (Hammond and Marshall, 1925). In one animal the uterus had undergone pseudo-pregnant development, although the ovary contained no corpora lutea, only blood follicles. This condition was found to be associated with a well-luteinised granulosa round the periphery of the blood follicles. The mammary glands of this animal (a virgin) were quite abnormal, the ducts, though short and lacking well-developed acini, being gorged with milk.

VII. *Summary.*

1. Extracts containing the gonad-stimulating principle of the anterior pituitary body were prepared from urine of pregnancy by a method similar to those employed by Zondek and Aschheim and by Evans and Simpson.

2. Ovulation was produced freely in non-copulated oestrous rabbits by one intravenous injection of these preparations. It has not so far been possible to correlate the number of follicles ovulating, or even the occurrence of ovulation, with the dosage given.

3. Following ovulation induced by this means corpora lutea develop and function without extraneous assistance; the result being a period of pseudo-pregnant development in the uterus and mammary glands. Pseudo-pregnancy thus induced is of the normal length, and has the usual characteristic features.

4. The fact that corpora lutea develop after ovulation induced without copulation shows that the stimulus to luteinisation of the ruptured follicle is initiated by the actual act of ovulation and not as a delayed effect of copulation.

## DESCRIPTION OF PLATE 1.

FIG. 1.—Ruptured follicle 15-30 hours after injection of urine extract, showing ovulation point filled by blood clot. (APF2 r.t.)  $\times 45$ .

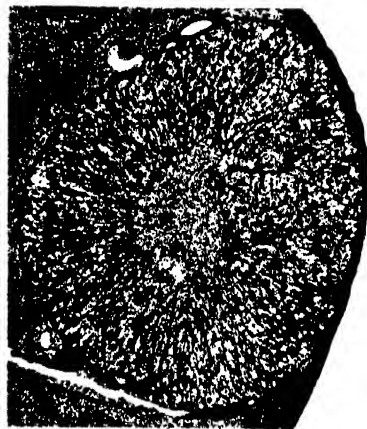
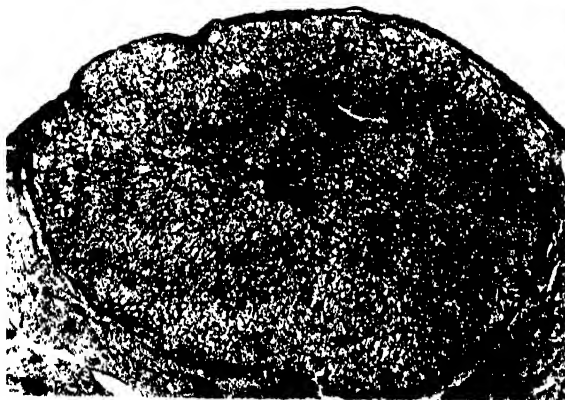
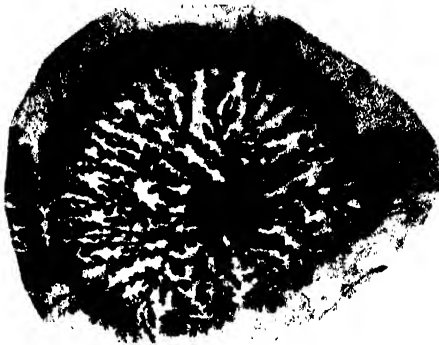
FIG. 2.—Corpus luteum 10 days after injection resulting in ovulation. Normal fully-functional corpus luteum. Median section. (APF20,)  $\times 25$ .

- FIG. 3.—Corpus luteum 20 days after injection resulting in ovulation, showing beginning of atrophy. Median section. (APF10.)  $\times 25$ .
- FIG. 4.—Mammary gland 10 days after ovulation induced by injection in a virgin animal showing pseudo-pregnant condition of active proliferation. (APF20.)  $\times \frac{1}{2}$ .
- FIG. 5.—Mammary gland 20 days after ovulation induced in a virgin animal, showing pseudo-pregnant development undergoing atrophy. (APF10.)  $\times \frac{1}{2}$ .
- FIG. 6.—Uterine mucosa 10 days after induced ovulation, showing pseudo-pregnant development. (APF20.)  $\times 15$ .

*Photomicrographs by Mr. F. J. Pittork.*

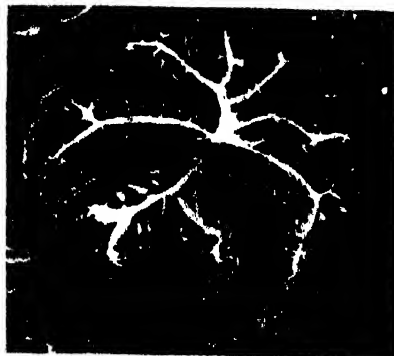
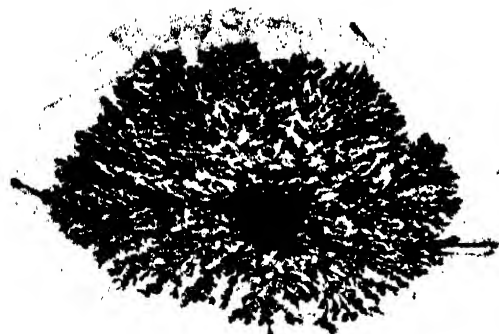
#### REFERENCES.

- Ancel and Bouin, 1909a. 'C. R. Soc. Biol.,' vol. 66, p. 680.
- Ancel and Bouin, 1909b. 'C. R. Soc. Biol.,' vol. 67, p. 466.
- Asdell, 1926. "Thesis for Ph.D. Cambridge."
- Bellerby, 1928. 'Lancet,' vol. 214, p. 1168.
- Bellerby, 1929. 'J. Physiol.,' vol. 67, p. xxxiii.
- Evans, 1924. "Harvey Lectures."
- Evans and Simpson, 1929. 'Proc. Soc. Exp. Biol., Med.,' vol. 26, p. 595.
- Fee and Parkes, 1929. 'Jour. Physiol.,' vol. 67, p. 384.
- Friedman, 1929a. 'Amer. J. Physiol.,' vol. 89, p. 438.
- Friedman, 1929b. 'Amer. J. Physiol.,' vol. 90, p. 617.
- Hammond and Marshall, 1914. 'Proc. Roy. Soc.,' B, vol. 87, p. 422.
- Hammond and Marshall, 1925. "Reproduction in the Rabbit."
- Heape, 1905. 'Proc. Roy. Soc.,' B, vol. 76, p. 260.
- Marrian, 1929. 'Biochem. J.,' vol. 23, p. 1233.
- O'Donoghue, 1913. 'J. Physiol.,' vol. 46, p. vi.
- Parkes, 1929. 'Proc. Roy. Soc.,' B, vol. 104, p. 171.
- Smith and Engle, 1927. 'Amer. J. Anat.,' vol. 40, p. 129.
- Teel, 1926. 'Amer. J. Physiol.,' vol. 79, p. 170.
- Zondek and Aschheim, 1927. 'Arch. Gynaek.,' vol. 130, p. 1.
- Zondek and Aschheim, 1928. 'Klin. Wochen.,' vol. 7, p. 831.



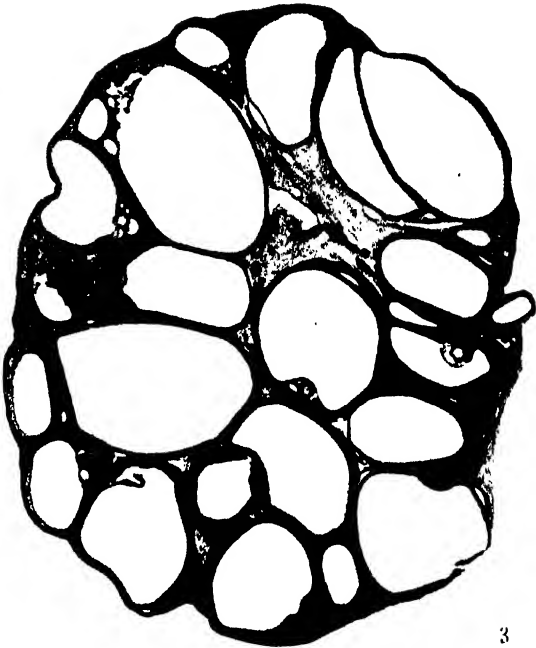
2

3

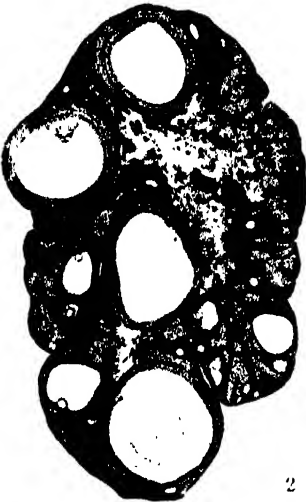




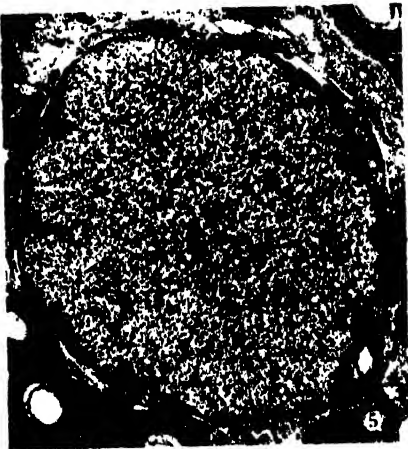
1



3



2



5



4

*On the Relation between the Anterior Pituitary body and the Gonads.—  
Part II. The induction of ovulation in the aëstrous ferret.*

By MARGARET HILL and A. S. PARKES (Beit Memorial Research Fellow),  
University College, London.

(Communicated by C. L. EVANS, F.R.S.—Received June 3, 1930.)

[PLATES 2-4.]

*I. Introduction.*

The factors responsible for the alternation of reproductive activity and aëstrous quiescence are imperfectly known, but in view of much recent work on the regulation of the ovary (Smith and Engle, 1927, Zondek and Aschheim, 1927), it is necessary to suppose that the anterior pituitary body is involved. On such a view aëstrus might be caused by inability of the ovary to respond to stimulation from the hypophysis, but it is more reasonable to suppose that the onset of the breeding season is due to increased activity of the anterior pituitary body, and that aëstrus results from decreased activity. In the circumstances it seemed that results of interest might be obtained by the administration of preparations containing the gonad-stimulating principles of the anterior pituitary body to animals during aëstrus. Most of the common laboratory animals have no definite aëstrus, and the choice of an animal for work on aëstrus is almost limited to the ferret or the dog. Of these, the former is obviously more suitable, especially as a considerable amount is now known about its reproductive processes.

The work of Marshall (1904), Robinson (1918) and Marshall and Hammond (1930) has made it possible to make the following statements regarding the aëstrous cycle in the ferret:—

- (a) The breeding season is restricted and lasts only from April to August. During the remainder of the year the reproductive organs are in a state of quiescent aëstrus.
- (b) The ferret, ovulating like the rabbit only after coitus, remains in aëstrus persistently through the breeding season if mating does not take place. Aëstrus is characterised by a prominent swelling of the vulva, which is normally indicative of the presence of ripe follicles in the ovary.



- (c) Ovulation occurs about 30–40 hours after copulation, which, if fertile, results in a pregnancy of about 6 weeks. If copulation is infertile a period of pseudo-pregnancy occurs during which the development of the corpora lutea in the ovary causes extensive hypertrophy of the uterine endometrium and mammary glands. This period of pseudo-pregnancy lasts almost as long as true pregnancy and is followed by the return of œstrus, or, if late in the breeding season, by anœstrus.

The anœstrus from late August to March is remarkably definite, and the ferret is therefore well suited for work requiring a completely quiescent reproductive system. In addition, the swelling of the vulva makes it possible to diagnose the œstrous condition in the intact animal and is a valuable guide as to the endocrine activity produced in the ovary.

It was hoped, by coincident stimulation of the male, to cause the animals to breed during anœstrus, but failure to induce spermatogenesis rendered this impossible. The present paper is therefore devoted to an account of the changes produced in the anœstrous female by the preparations used.

## II. *Material and Technique.*

*Ferrets.*—The animals were obtained from dealers, with the exception of some few bred in the laboratory, but all were kept under observation for at least some weeks before being used for experiment. In order to reduce the number of animals required, two stages were sometimes obtained by the operative removal of one ovary (usually the left).

*Histological Technique.*—The ovaries were decapsulated before fixation, the capsule and fallopian tube being fixed separately. The ovaries were usually weighed, this being carried out in 70 per cent. alcohol during up-grading. Complete serial sections (7  $\mu$ ) were made of all ovaries. The vagina and uterus were cut into small pieces before fixation. Bouin's fluid was used as a routine and sections were stained in Ehrlich's hæmatoxylin and eosin.

Camera lucida drawings were made of the group of largest follicles in each ovary, and the mean diameter calculated from two measurements made at right angles.

*Extracts Used.*—The extracts were prepared either from ox pituitaries or from urine of pregnant women. Tissue extracts were made by (a) the alkali method described by Teel (1928), or (b) the acid method of Bellerby (1929a). The urine extracts were made by the process described in Part I of the present

series of papers, in which the details of the fractional precipitation with alcohol will also be found. The final concentration of the urine extracts was 1 c.c. = 30 c.c. original urine. In the absence of definite information as to the keeping properties of the extracts, one extract was not used for more than 4-5 days. All injections were made subcutaneously, in amounts of 5 c.c. per day.

### III. *Experimental Results.*

*Size of Ovary, Follicles and Corpora Lutea in Normal Ferrets.*—The data for normal ferrets required for comparison with Table I are as follows. Hammond and Marshall found the diameter of anæstrous follicles to be from 0.24 mm. to 0.72 mm. according to the proximity of the breeding season. Robinson's figures for the volumes of the anæstrous follicle indicate a similar result. In the present experiments, control anæstrous animals killed during November had an average diameter of about 0.30 mm. During œstrus, the mature follicle has a diameter of about 1.30 mm. according to Hammond and Marshall, while Robinson's figures, though indicating a wider range, have about the same average. Our own data for the normal follicle during œstrus show an average diameter of 1.20 mm. to 1.30 mm. The diameter of the largest corpora lutea during pseudo-pregnancy is given by Hammond and Marshall as 2.08 mm. The weight of the whole ovary does not seem to have been noted, but in the present experiments a pair of normal anæstrous ovaries was found to average about 50 mgm. This weight must vary considerably according to the previous history of the animal.

*Summary of Experiments.*—Twenty-one female ferrets, 5 months or more old, were used between the end of September and the end of January. Under the conditions prevailing, no spontaneous œstrus was observed in normal animals between the beginning of August and the end of April, so that there can be little doubt that the effects observed in treated animals were due to injection. Urine extracts were used on 18 of the animals, while one received an acid extract and two received alkali extracts of ox anterior pituitary tissue. Of the 18 animals treated with urine extract, 6 received the "total water-soluble fraction," and the remainder fractional alcohol extracts as shown in Table I. (See Part I of these papers.) Ovulation was obtained only with the 30 per cent. alcohol soluble fraction; six of the eight animals receiving this particular extract showed ruptured follicles, tubal ova, or normal corpora lutea. Other fractions and extracts, however, completely failed to cause ovulation, cystic or luteinising follicles only resulting.

Table I.—Administration of Anterior Pituitary Preparations to the Anæstrous Ferret.

Number of animal.	Injection.		Time of operation or autopsy. Days after first injection.	Weight of ovary(ies) (gm.).	Condition of ovary(ies).	Condition of vulva.	Condition of uterus.
	Type of extract.	Days injected.					
44	30 per cent. alc. sol.	10	11	0.143 (both)	Ovulated. Many ruptured follicles	Vulva swelling at 3 days	Early æstrus.
45 (left ovary)	"	9	10	Left ovary not weighed	"	"	"
45 (autopsy)	"	10	11	0.112 (right only)	"	"	"
49	"	10	18	—	Undeveloped. Little effect produced	—	—
46	"	10	24	—	Good corpora lutea (diameter = 1.10 mm.)	Slight swelling	Slight development.
48	"	10	31	—	Good corpora lutea (diameter = 1.32 mm.)	Swelling at 3 days, down at autopsy	Early pseudo-pregnant.
47	"	10	52	—	No corpora lutea. Undeveloped	"	"
64 (left ovary)	"	13	14	0.119 (left only)	Good corpora lutea	"	Slight development.
64 (autopsy)	"	17	18	0.101 (right only)	Good corpora lutea (diameter = 1.50 mm.)	—	—
65 (left ovary)	"	13	14	0.133 (left only)	Good corpora lutea	Swelling at 5 days, down at autopsy	Early pseudo-pregnant.
65 (autopsy)	"	13	18	0.096 (right only)	Good corpora lutea (diameter = 1.30 mm.)	—	—
53	50 per cent. alc. sol.	6	8	—	Large number of cystic follicles	As ferret 64	Early pseudo-pregnant.
54	"	6	8	—	"	Completely swollen	Æstrus.
55	"	6	8	—	"	"	"
					(av. diameter = 1.88 mm.)	"	"

	Total H <sub>2</sub> O sol. ....	S				
59	..	0-120 (both)	Little effect. A few small follicles becoming cystic	Slight swelling	—	—
14	..	0-079 (both)	No development	Large swelling at 10 days	Early oestrus.	—
66	..	0-089 (both)	"	Little growth	Some development	—
61	..	0-469 (both)	Large cystic follicles	Moderate swelling	—	—
41 (left ovary)	..	0-097 (left only)	Large follicles becoming cystic	Good swelling at 12 days	—	—
41 (autopsy)	..	0-021 (right only)	Small cystic follicles	Going down	Oestrus.	—
60 (right ovary)	..	0-136 (right only)	Follicles becoming cystic	Good swelling at 12 days	—	—
60	..	0-042 (left only)	Small cystic follicles	Going down	—	—
(autopsy)	..	0-085 (left only)	"	Moderate swelling	—	—
62	Ant. pit. tissue (acid)	0-039 (right only)	Cystic follicles	Down	—	—
62	..	0-061 (both)	No appreciable change	No change in vulva	—	—
63 (autopsy)	.. (alk.)	0-124 (both)	Large cystic follicles. One blood follicle	Good swelling	—	—
42	.. (acid)					

*Types of Ovarian Reaction.*—It will be seen from the above records that the reaction of the ferret ovary to preparations containing the gonad-stimulating principles of the anterior pituitary body differs somewhat from the response found in other mammals. In mice the alkali extract of ox anterior pituitary tissue commonly produces complete luteinisation of existing follicles, while in the ferret this type of preparation had no such decisive result. In fact, no healthy solid corpora lutea atretica formed without ovulation (as judged by a contained ovum) have been found in these experimental ferrets. Judged by its endocrine effect, a criterion emphasised by Wiesner and Crewe (1930), the ovarian reaction of the ferret to the NaOH extracts is also different in so far as oestrous changes appear in the vagina, indicating the secretion of oestrin by the ovary. In the mouse, on the other hand, this effect does not usually predominate.

The ferret also appears to differ from the mouse in the response to urine extracts. The large blood follicles produced in the latter are completely missing in the ferret, while the cystic type of follicle found in the ferret is rare in the mouse. Difference is also found in that the reaction of the individual follicles of one pair of ovaries is more constant. In the mouse, with urine extracts, mature follicles, atretic corpora lutea and blood follicles may occur side by side, a condition not found to the same extent in the ferret.

As with similar work on other mammals, much individual variation is found in response to the same dosage of the same extract. Ferret 49, and probably 47 also, failed to respond appreciably to treatment resulting in ovulation in four other animals. Such variation, however, is no more striking than that found in work with oestrin.

*Histology of the Ovarian Reaction.*—When injection leads to ovulation the usual maturation changes appear to take place quite normally. This applies not only to the general preliminary growth of the follicle, but also to the more special changes which immediately precede ovulation, and which only occur in the normal animal after coitus. Thus, induced ovulation is preceded by the formation of the corona radiata (see Plate 4, fig. 1). Such abnormalities of ovulation as are observed (retention of ovum after rupture, etc.) appear to be due to the overcrowding together of large numbers of ovulating follicles. The ovaries of ferret 45, for instance, contained 40–50 ruptured follicles (Plate 4, fig. 2). After ovulation an abnormally large amount of liquor folliculi tends to be retained in some instances. Healthy corpora lutea develop after induced ovulation, even in the absence of further extraneous stimulation by injection (Plate 2, fig. 5, and Plate 3, fig. 5). It has not, however, been

ascertained whether such corpora lutea have the normal length of functional life.

The large abnormal follicles found in certain of the animals, notably ferrets 53-55 (Plate 2, fig. 3), appeared to have lost all power of ovulating and to be definitely cystic. The chief abnormal features presented by these follicles were (a) large size compared with normal mature follicles, (b) separation of the follicular granulosa from the theca, and general unhealthy appearance of the granulosa, which may be drawn out to form a lining membrane, (c) absence of any signs of corona radiata or other sign of normal maturation, and (d) atretic changes in the ovum. No tendency to form blood follicles was found in these cystic ovaries. There is no probability that these follicles would ever have ovulated. Where such follicles were present in considerable numbers, very large ovaries resulted.

In addition to completely cystic follicles, others exhibiting various degrees of a type of "luteinisation" were found. These in most cases were of considerable size, showing that coincident growth had also taken place. These follicles differed greatly, however, from the luteinising follicles found in the immature mouse after injection of similar preparations. In the mouse the granulosa undergoes true luteinisation and pushes vigorously into the antrum, which ultimately becomes filled up. A solid corpus luteum is thus formed, which is only distinguished from a corpus luteum vera by the inclusion of the remains of the ovum. In the ferret, on the other hand, the tissue enlarging is the theca interna, while the granulosa is degenerating and shows no sign whatever of growing into the antrum or of luteinisation (Plate 4, figs. 5 and 6). In the more advanced cases the granulosa is stretching out to form a membrane lining the antrum. Such follicles appear to represent early stages in the formation of the cystic follicles described in the preceding paragraph. In these ferrets no unruptured follicles have been found showing an actively luteinising granulosa growing into the antrum; in other words, no early stage in the formation of corpora lutea atretica have been found. It would thus appear that the processes which lead in the mouse to the formation of healthy corpora lutea without ovulation, cause in the ferret the formation of cystic follicles.

*Effects on the Uterus.*—The secondary effects on the uteri were much as expected. In most animals considerable enlargement had taken place, the mucosa showing various stages of the folding and gland proliferation typical of oestrus (Plate 3, fig. 3).

Oestrous changes in the uterus were found associated with cystic and

luteinising follicles, as well as with mature follicles. In the second case the output of œstrin by the ovary presumably exceeded that of the luteal hormone, if any. Where corpora lutea were formed following ovulation, early pseudo-pregnant changes of the type described by Hammond and Marshall were observed in the uterus (Plate 3, fig. 4). Whether these changes would ever have become complete is uncertain; the animal designed to show this failed to ovulate. It seems probable, however, that implantation would have taken place in these animals had fertilisation been effected.

*Effect on the Vagina and Vulva.*—With two exceptions, all the animals showed considerable growth of the vulva, but in many cases the swelling was much below that normally occurring at œstrus. All the animals which ovulated, however, showed vulval development approaching that normally found, as did those with many cystic follicles. Vulval swelling, indicative of œstrin production, was observed in animals showing no apparent follicular growth. This again emphasises that the anterior pituitary substances can cause the production of ovarian hormones in the absence of the structures normally associated with them. Nine animals from which vaginal smears were made during induced œstrus, all showed the cornification of the vaginal epithelium typical of normal œstrus (Plate 4, figs. 3 and 4). Following induced ovulation the vulval swelling subsided rapidly whether or not injection was continued.

In two animals (64 and 65) copulation took place with a treated male, though the aspermia of the male rendered the act necessarily infertile. This provided evidence, however, that the induced vulval and vaginal development was adequate and that the usual psychical symptoms of œstrus appeared.

#### IV. Discussion.

It is obviously desirable that purely theoretical discussion of the problem of the anterior pituitary body should be avoided so far as possible, but it is necessary to mention four points in connection with the experiments described above.

*Formation of Corpora Lutea.*—In two of the animals described, corpora lutea which appeared histologically to be sound, and which were certainly functionally active, were formed after ovulation without further injection. This is entirely in keeping with the results on rabbits reported in Part I. Of the two possible explanations (a) that the injection or the act of ovulation stimulates the anœstrous pituitary to exert the luteinising stimulus, (b) that the corpus luteum can develop in the absence of pituitary stimulus, the latter seems unlikely,

though in view of results reported elsewhere (Deanesly, Fee and Parkes, 1930) it cannot be entirely ignored.

*Regulation of the Anterior Pituitary Cycle.*—The experiments recorded above make it at least highly probable that the commencement of the breeding season in spring and its end in late summer are due to changes in the anterior pituitary body. Some kind of cycle must, therefore, occur in the anterior pituitary quite apart from any changes during the breeding season itself. It is necessary, therefore, to ask what factors are responsible for the stimulation of the pituitary body about April. In view of the known dependence of the breeding season in other mammals on environmental conditions, it would be natural to look to some such agency in the ferret. It is indeed difficult, however, to imagine what environmental changes take place during April in an artificially heated animal house not particularly accessible to direct sunlight. As an experimental approach to the problem, two male and two female ferrets were exposed daily for 10 days during November to 5 minutes ultra-violet light. No changes of any kind, however, were observed in their genitalia. It must be admitted that the factors activating the anterior pituitary body at the beginning of the breeding season are obscure for the moment.

*Mechanism of Ovarian Regulation.*—The experiments on the ferrets throw no definite light on the problem of whether the whole of the regulation of the ovarian cycle is carried out by one anterior pituitary substance, having a different effect in different concentrations (as originally supposed by Zondek and Aschheim, 1927), or whether two are concerned (as supposed by Bellerby, 1928), and Wiesner and Crew, 1930). So far as this ferret work goes, the latter view seems more likely, in which case the dual effects produced in many of the animals would be due to the simultaneous injection of both substances.

*Rôle of Copulation in causing Ovulation.*—Finally, it remains to point out that, since both the pre- and post-insemination maturation changes of the follicle can be brought about by injection, copulation presumably causes ovulation as the result of reflex stimulation of the anterior pituitary body. On this view the pituitary body attains a certain level of activity and produces mature follicles as found during the persistent oestrus and only attains the higher level of activity required to cause ovulation after the stimulus provided by copulation. Such a state of affairs clearly exists in the rabbit, where copulation can also be replaced by the extraneous addition of anterior pituitary substance (Bellerby, 1929b) and where hypophysectomy immediately after copulation inhibits ovulation.



V. *Summary.*

1. Ovulation may be induced in the anoestrous ferret by the administration of certain preparations containing the gonad-stimulating substance or substances of the anterior pituitary body. Other preparations, however, resulted in the formation of large cystic follicles or in the coincident growth and partial luteinisation of follicles.

2. In all cases where ovulation occurred, as well as in many others, the anoestrous accessory organs (uterus, vagina and vulva) underwent the changes typical of oestrus as a result of the ovarian stimulation.

3. Following ovulation, corpora lutea develop and function with or without further administration of extracts. In correlation with this ovarian development, pseudo-pregnant changes take place in the uterus.

4. Owing to failure to induce spermatogenesis in the male, pregnancy was not obtained during anoestrus, though copulation occurred between treated males and treated females. There can be little doubt, however, that the ova from the induced ovulations were fertilisable, and that implantation could have taken place.

Our grateful thanks are due to Prof. C. Lovatt Evans, F.R.S., for his facilitation of the work described in this series of papers. During the course of the experiments we have been greatly assisted by discussions with Dr. F. H. A. Marshall, F.R.S., and Mr. J. Hammond. To Prof. J. P. Hill, F.R.S., we are indebted for histological accommodation, and also to Dr. F. W. R. Brambell for much histological advice. The urine of pregnancy was obtained through the kindness of Prof. F. J. Brown.

The expenses of the research have been defrayed from a grant to A.S.P. from the Medical Research Council.

DESCRIPTION OF PLATES 2-4.

*Guide Letters.*—C., cornified cells; L.G., membrana granulosa undergoing luteinisation; M.G., membrana granulosa; O.P., ovulation point; T.E., theca externa; T.I., theca interna; V.E., vaginal epithelium.

PLATE 2.

FIG. 1.—Ovary of normal anoestrous ferret.  $\times 13$ . Compare figs. 2, 3, 4 at the same magnification.

FIG. 2.—Ovary of normal oestrous ferret, showing size of mature follicles.  $\times 13$ .

FIG. 3.—Ovary of ferret 55, showing large cystic follicles, after 6 days injection of urine preparations.  $\times 13$ .

FIG. 4.—Ovary of ferret 64 after 17 days injection. Normal corpora lutea formed after ovulation.  $\times 13$ .

FIG. 5.—Ovary of ferret 42, 21 days after the last injection, showing corpus luteum formed after ovulation without further injection.  $\times 13$ .



2



3



4



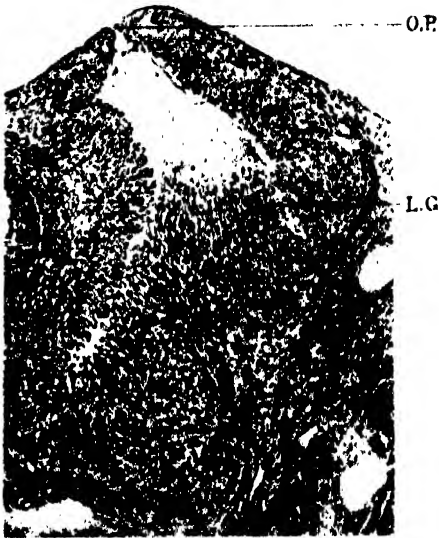
4



5



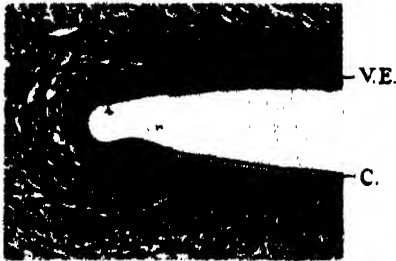
1



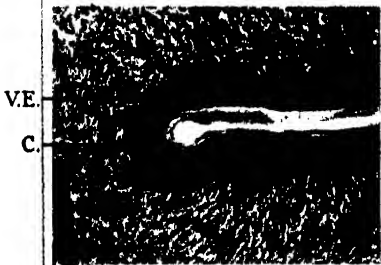
2



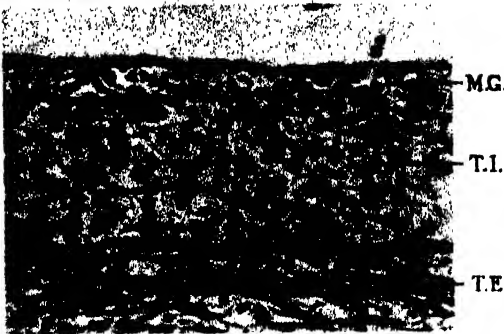
5



3



4



6

PLATE 3.

- FIG. 1.—Uterus of normal anoestrous ferret (non-parous).  $\times 20$ .  
 FIG. 2.—Uterus of normal oestrous ferret.  $\times 20$ .  
 FIG. 3.—Uterus of ferret 53 (anoestrous), after 6 days injection, resulting in the production of cystic follicles in the ovary, showing early oestrous changes. (See Plate 2, fig. 3.)  $\times 20$ .  
 FIG. 4.—Uterine endometrium of ferret 48, showing condition of early pseudo-pregnancy correlated with the presence of corpora lutea in the ovary. (See Plate 2, fig. 5.)  $\times 55$ .  
 FIG. 5.—Ovary of ferret 46, showing corpora lutea 14 days after last injection.  $\times 25$ .

PLATE 4.

- FIG. 1.—Tubal ova of ferret 45.  $\times 75$ .  
 FIG. 2.—Ruptured follicle in ferret 45, showing ovulation point partially occluded by a blood clot.  $\times 50$ .  
 FIG. 3.—Vagina of normal oestrous ferret, showing cornified epithelium.  $\times 55$ .  
 FIG. 4.—Vagina of ferret 54, after 6 days' injection, showing oestrous cornification.  $\times 55$ .  
 FIG. 5.—Wall of mature follicle in normal oestrous ferret.  $\times 250$ .  
 FIG. 6.—Wall of follicle of ferret 41, showing "luteinisation" of the theca interna and degeneration of the granulosa.  $\times 250$ .

*The photographs and photomicrographs are the work of Mr. F. J. Pittock.*

REFERENCES.

- Bellerby, 1928. 'Lancet,' p. 1108.  
 Bellerby, 1929a. 'J. Physiol.,' vol. 67, p. xxxii.  
 Bellerby, 1929b. 'J. Physiol.,' vol. 67, p. xxxiii.  
 Deaneely, Fee and Parkes, 1930. 'J. Physiol.' (in press).  
 Hammond and Marshall, 1930. 'Proc. Roy. Soc.,' B, vol. 105, p. 607.  
 Marshall, 1904. 'Quart. J. Micr. Sci.,' vol. 48, p. 323.  
 Robinson, 1918. 'Trans. Roy. Soc., Edinburgh,' vol. 52, p. 303.  
 Smith and Engle, 1927. 'Amer. J. Anat.,' vol. 40, p. 159.  
 Teal, 1926. 'Amer. J. Physiol.,' vol. 79, p. 170.  
 Wiener and Crew, 1930. 'Proc. Roy. Soc., Edinburgh,' vol. 50, p. 29.  
 Zondek and Aschheim, 1927. 'Arch. Gynaec.,' vol. 130, p. 1.

## *A Cytological Demonstration of "Genetic" Crossing-Over.*

By C. D. DARLINGTON, John Innes Horticultural Institution, Merton.

(Communicated by Sir Daniel Hall, F.R.S.—Received July 1, 1930.)

[PLATE 5.]

### 1. *Introduction.*

A stage in meiosis where the paired chromosomes form loops, coming together at points distributed at random along their length, was first described by Rückert (1892) in *Pristiurus*. These points we now recognise in suitable material as exchanges of partner amongst the four pairing half-chromosomes. But although Rückert did not appreciate this detail of structure he grasped the possible significance of their relationship: "Die bei der Reifung der Geschlechtzellen eintretende Verklebung vorhergetrennter Chromosomen bezweckt einen Substanztausch derselben." He considered that here was the possibility of a chromosome "amphimixis," carrying a step farther Weismann's idea of nuclear "amphimixis."

Similarly Correns (1902) and Boveri (1904, contrary to his earlier view) foresaw the possibility of different parts of a chromosome behaving independently at meiosis. The discovery of linkage by Bateson, Saunders and Punnett in 1905 was a verification of these predictions, although not at first interpreted in this sense.

Again in 1909, Janssens pointed out the possibility of exchanges at such points of contact as those seen by Rückert. The hypothesis that exchanges of segments actually take place at these points provided the interpretation which Morgan (1911) and his school have used to show the linear arrangement of the hereditary materials. And their success is the highest testimony to the validity of Janssens' assumptions.

While logical construction on the cytological side has anticipated genetical observations, cytological observations affecting this question have lagged far behind. Before considering the present observations it will be necessary to review the theories relating to the two and give an outline of the chromosome behaviour which is known to determine segregation and is therefore supposed to be concerned with crossing-over. This description will define the use of terms.

2. *Chromosome Behaviour at Meiosis.*

Homologous chromosomes (each a single thread) associate in pairs laterally, particle by particle, at the prophase of meiosis. The double (pachytene) thread formed in this way splits longitudinally, giving a quadruple thread consisting of four half-chromosomes or "chromatids." Splits appear at diplotene which separate pairs of chromatids, but the associations of pairs are not continuous throughout: the chromatids change partners. A point at which this change occurs I shall refer to as a "chiasma," because the name describes the characteristic crossing of two of the four chromatids. I will, therefore, define a chiasma as *The occurrence of a single exchange of partner in a system of four chromatids associated in pairs.*

There is no direct evidence as to what causes the chiasma, but there are two opposed theories to account for it (fig. 1). The first is that it is the result of two splits meeting, the one equational, *i.e.*, separating identical chromatids, the other reductional, *i.e.*, separating the original somatic chromosomes as they came together. This is the view to which the majority of cytologists incline, though merely for the reason that it involves the fewest obvious cytological assumptions. It assumes no change in the identity of the chromatids because no such change is observed (Robertson, 1916; Wenrich, 1916; Wilson, E. B., 1925; Seiler, 1926; McClung, 1927; Bělař, 1928).

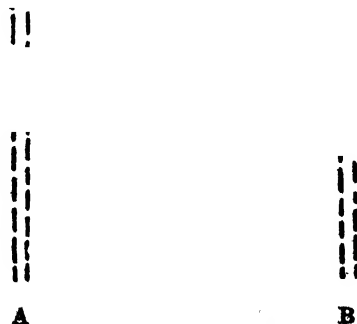


FIG. 1.—Genetical interpretations of chiasmata: A. Classical; B. Chiasmotype.

The second view is that put forward by Janssens (1909, 1924). According to Janssens, chiasmata result from a change in the linear individuality of two of the four chromatids, so that if the two original chromosomes were of the structure ABCDEF and *abcdef*, and a chiasma is formed in the length between the paired points Bb and Cc, then the structure of the four continuous threads is to be taken as ABCDEF, AB*cd*ef, *ab*CDEF and *abc*def. In this case it is assumed that identical threads are always associated at diplotene. This change in the linear individuality of two of the four chromatids (partial chiasmotypy) is only one of the assumptions that Janssens made. He also assumed that all four threads might interchange segments at the same point (total chiasmotypy). This would result, not in a chiasma (in my sense) but in an interlacing. Janssens also believed that other types of less definite association occurred amongst paired chromosomes at diplotene and later\* ("soudures dont le caractère semble plus transitoire"). Janssens attempted to show the validity of his assumptions directly and indirectly. His studies went far towards showing the physical structure of the chiasma after it was formed and they have been confirmed by the recent work of Belling (1925, 1926), Newton and Darlington (1927, 1929 and 1930) and Maeda (1930) in plants. His observations of discontinuity in the separating anaphase chromatids (confirmed by Bělař, 1928) and of their interlocking (cf. also Maeda, 1930) are indirect evidence of the origin of chiasmata that cannot be neglected.

The position now is that Janssens' assumptions are unproved. And since Janssens himself seemed to regard them as directly provable this lack of direct evidence has weighed against them. For example Robertson (not unreasonably) remarks (1915): "The formation of cross-overs as a result of the opening out process of the four strands does away with the possibility of a 'compénétration graduelle de deux chromosomes au niveau d'un chiasma avec la soudure des filaments qui se touchent les premiers.'" And Seiler (1926) concludes that "Auch bei den bescheidensten Ansprüchen an den erklärenden Wert einer Hypothese diejenige von Janssens den crossing-over Befunden niemals gerecht werden kann, ganz abgesehen davon dass sie keine Tatsachenbasis hat."†

\* Amongst these are what I should call "terminal chiasmata" (1929b).

† Other criticisms are less reasonable (cf. Darlington, 1930). For example, McClung (1927a) remarks that "Janssens quite overlooks the most important circumstances in the history of the meiotic chromosome, i.e., the simultaneous existence of two planes of division at right angles to each other proceeding from opposite ends of the chromosome." This begs the whole question, for it accepts Wenrich's hypothesis (1916) of what happens in *Phrynotettix* as applicable to *Mecostethus*, which Janssens (1924) and, less definitely, McClung (1927b) have shown not to be the case, for in *Mecostethus* which they have studied pachytene pairing is said to be incomplete.

But the agreement of Janssens' hypothesis with the genetical results of which it was to a great extent a prediction (*cf.* Belling, 1928; Darlington, 1930*b*) is at least as important an objection to the ordinary cytological hypothesis as the lack of particular observations is to Janssens' hypothesis.

The question therefore arises as to whether they could ever be demonstrated directly, and in my opinion this is in the last degree improbable. It is to be expected that an instantaneous change in relationship occurring between four indistinguishable and closely paired threads (each less than 100  $m\mu$  in diameter) is responsible for the changes that Janssens and the geneticists have imagined. Direct observation of such an occurrence is inconceivable. Surely then we ought to consider that Janssens' "chiasmotypy" and its alternative, the classical diplotene hypothesis are equally assumptions, equally incapable of direct demonstration and equally requiring demonstration by inference from material in which we can distinguish the results of the two hypotheses.\*

I conclude that the assumption of "partial chiasmotypy," which demands crossing-over between two of the four chromatids at every chiasma, is the only possible working hypothesis for the correlation of the cytological and genetical observations. I have lately attempted to show (1930*b*) that there is no insuperable obstacle to this hypothesis in the genetical and cytological observations on *Drosophila* or *Primula*.

In considering the new cytological observations it must be remembered that the confusion in Janssens' thesis between the observations of chiasmata and the supposed evidence of their cause is unnecessary. My earlier hypothesis (1929*a* and *b*), that *chiasmata are the condition of chromosome pairing and of meiosis*, is distinct from the revised version of Janssens' hypothesis that I am now adopting (1930*b*) to the effect that *chiasmata are conditioned by crossing-over between two of the conjugant chromatids at pachytene*.† I therefore use the term *chiasma*, as above, in a morphological sense and without the genetic implications of Janssens (with which it is usually connected).

### 3. The Crucial Test.

Belling (1929) and Newton and Darlington (1929) have independently examined the evidence of configurations in triploids, both knowing that association occurs between pairs of chromosomes at pachytene. Belling has considered that the occurrence of certain configurations (similar to those

\* See Appendix and Darlington (1930*b*).

† A third hypothesis suggesting *how* crossing-over might take place (Belling, 1928) is likewise independent of the other two. They stand or fall separately.



described later in tetrasomic forms) was conclusive evidence of crossing-over. We considered that the presence of the third thread at pachytene prevented a final conclusion that associations between all three threads could not take place at one point (perhaps successively). In the tetraploid no such doubt can exist. And since in tetraploids I found no configurations of these types (1929a) I reached a negative conclusion. The present observations are the result of a special search for the missing configuration in this critical material.

At pachytene in tetrasomic hyacinths the chromosomes come together in pairs as in disomic forms. But coming together at random they associate differently at different points; they therefore exchange partners (diagram model, Plate 5, fig. 12, and Darlington, 1929a, Plate 6, figs. 13-17). Although the intervening stages between pachytene and metaphase have not been studied (not being suitable for critical observation), the metaphase configurations (in disomic and tetrasomic forms) show the characteristic forms of chromosomes which have not undergone change since diplotene. Moreover the same types of configuration are found in tetraploid *Primula sinensis* at diplotene (Darlington, 1930b). That is, the chiasmata are distributed at random along the associated chromosomes. Since the chromosomes were assorted in pairs at random at pachytene the metaphase chiasmata are distributed at random amongst the different possible pairs of chromosomes.

Before studying the relations of these chiasmata to one another let us examine the possibilities under the two hypotheses, the classical and the chiasmatype. It is clear that configurations with simple exchanges are in accordance with both hypotheses. Similarly configurations where four chromatids engage in two chiasmata between chiasmata in which two of them engage with a third pair may be interpreted in both ways and are therefore inconclusive (Darlington, 1929a, p. 43). Such configurations have been illustrated. But there is a type of triangular configuration (such as has been found freely in triploid Tulipa and Hyacinthus, where the evidence of pachytene association is not unassailable, for the third free thread might be supposed to interfere with the paired threads) the occurrence of which would be a crucial test. The essential part of such a configuration is illustrated in the following diagram (fig. 2).

*Analysis of Chiasmata.*—The four chromatids of chiasma A have three in common with those of chiasma B (on either hypothesis). But the chromatids of one chiasma can only be derived from the four chromatids of two chromosomes. The fourth chromatid in A or B cannot be derived from a third chromosome, therefore the chiasmata A and B have all four chromatids in common.

If identical threads are associated at A and B, i.e., on both sides of the chiasma C, the change of pairing at C must be accompanied by an exchange of identity between two of the chromatids at C.

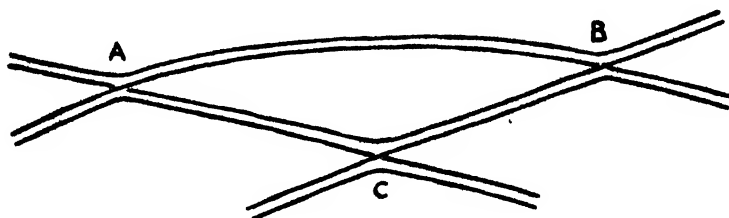


FIG. 2.

#### 4. The Demonstration.

Such configurations occur in tetrasomic *Hyacinthus* and *Primula sinensis*. They are usually too complex to illustrate without models and the types taken (figs. 3-6) are the simplest (and probably rarest) found. The interpretation (figs. 7-10) is in accordance with the conclusions of Belling (1927) and myself (1929) that "nodes" where four chromatids come together are chiasmata. This conclusion cannot always be directly verified, but in the case chosen for illustration with models (fig. 6, diagram fig. 10, models figs. 11 and 13) the interpretation of critical chiasmata is not disputable. Fig. 3 provides the simplest example of the critical triangular configuration; figs. 4, 5 and 6 provide compound examples.

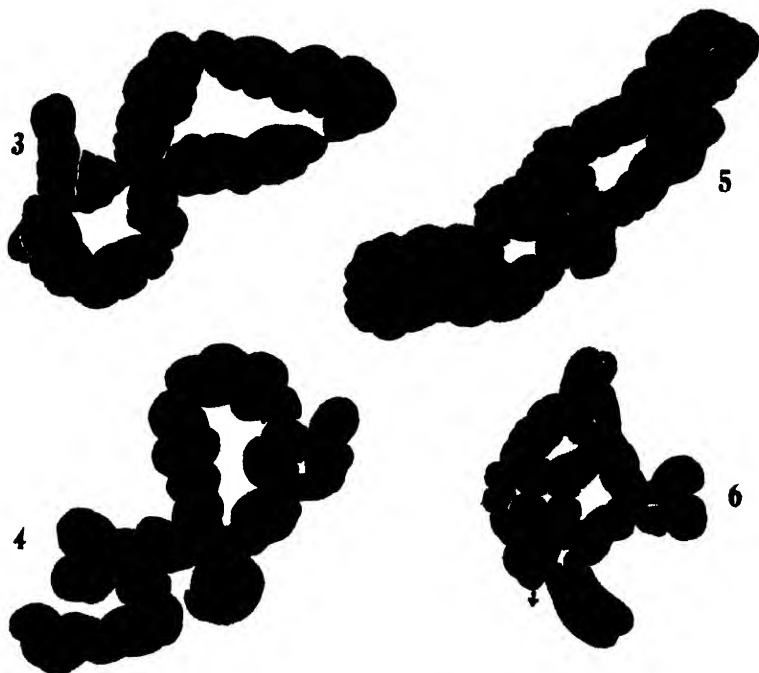
The models (Plate 5, figs. 11-13) illustrate the interpretation of fig. 6 and show its incompatibility with the classical hypothesis. On this hypothesis chiasmata have to be represented as derived from chromatids of three or four original chromosomes. This assumption would be contrary to the observations at pachytene. In view of these considerations, the observations are only compatible with the assumption that *where a chiasma is formed amongst four chromatids an interchange of segments (genetic crossing-over) has occurred between two of them.*

#### Summary and Conclusion.

Chromosomes associate in pairs at the prophase of meiosis in polyploids. A chiasma is the occurrence of a visible change of partners amongst four half-chromosomes. It follows therefore that, where two chromatids, which form a chiasma with two other chromatids, also form chiasmata with a third pair of chromatids on either side of the first chiasma, then the chromatids must be assumed to be in the same combinations on the two sides of this chiasma.

In such cases genetic crossing-over (i.e., an exchange of linear identity) must have taken place at the first chiasma.

Configurations of this kind occur in polyploid hyacinths and *Primula* and make it extremely probable that wherever an exchange of partners occurs



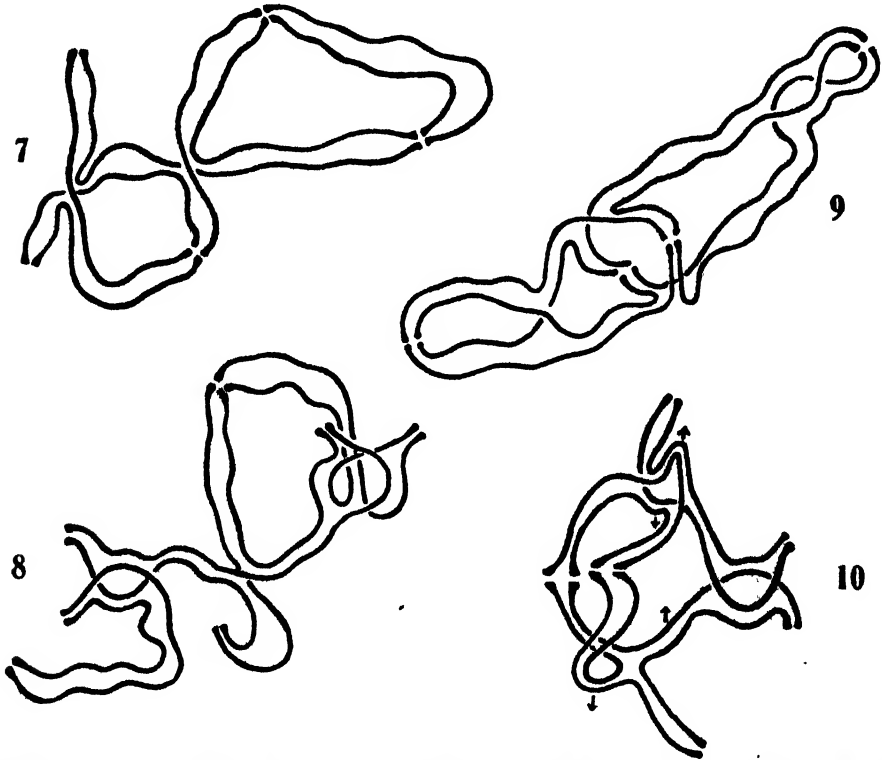
FIGS. 3-6.—Quadrivalents from first division metaphases in the pollen mother-cells of the tetrasomic hyacinth "La Grandesse." ( $2n = 28$ )  $\times 5300$  (drawn at 6400).

amongst four paired chromatids, crossing-over has occurred between two of them (Janssens' partial chiasmatypy). This conclusion is distinct from the hypothesis that the pairing of chromosomes depends on the formation of chiasmata between them.

Taking this as a working hypothesis it will be possible to study crossing-over from a cytological point of view. This is particularly important in considering its relation to structural change in the chromosomes. For example, it is possible to regard segmental interchange between non-homologous chromosomes as the result of crossing-over between small, relatively translocated, segments. This crossing-over would be between definite loci and with a definite frequency determined by the length of the segments in question. Such is the requirements on the basis of my hypothesis for explaining the origin of

half-mutants (1929a). In a similar way all mutation in *Oenothera* not involving changes in chromosome-number can probably be explained in terms of crossing-over.

In these terms segmental interchange becomes a *secondary* structural change,



FIGS. 7-10.—Line diagrams to show chromatid structure of the quadrivalents. Arrows indicate points of attachment to the spindle in a quadrivalent seen in side view (figs. 6 and 10).

distinguished from sporadic *primary* structural changes by the regularity of its occurrence both in frequency and result. This question will be considered more fully at a later date.

#### APPENDIX.

There are other theories of crossing-over which need not be considered in detail. For example, Seiler's hypothesis of re-arrangement within multiple chromosomes does not seem to be generally applicable on either cytological or genetical grounds. Chromosome linkage in *Solenobia* like that in *Oenothera* seems to depend on special conditions of chromosome organisation. Nor need

Chodet's assumption (1925) of a new line of cleavage at anaphase be considered. It is contradicted by all the recent observations on material suitable for observing the relations of the chromatids at this and the earlier states (cf. Newton on *Tulipa*, Belling and Darlington on *Hyacinthus*, Maeda on *Vicia* and *Lathyrus*, Newton and Darlington on *Fritillaria*).

Similarly Janssens' total chiasmotypy (with which Belling's hypotheses, 1928, is to some extent involved) is no longer valid for the following reasons :

(i) It is contrary to the genetical observations of crossing-over in the four-strand stage by Bridges and Anderson (1925) ; (ii) experiments in *Drosophila* have frequently shown over 50 per cent. of offspring without any crossing-over over considerable lengths of chromosome (cf. Jennings, 1923). In this 50 per cent. no total chiasmotypy can have occurred, and whether partial chiasmotypy occurs as well, or not, there must be chromosomes pairing without either type of exchange. This is a difficult assumption *a priori*, but I am inclined to reject it particularly because it is incompatible with the view that chiasmata are the condition of pairing ; (iii) I have never found any evidence of the type of interlocking the appearance of which suggested total chiasmotypy to Janssens ; (iv) on the contrary, evidence of various kinds is accumulating to indicate that the only association of chromosomes is that conditioned by chiasmata, although I appear to be alone in stating this categorically (Belling, 1926, 1927, 1928 ; Newton, 1927 ; Darlington, 1929a, 1929b, 1930 ; Maeda, 1930a, 1930b).

*Note.*—Certain genetical work on mosses appear to disagree with the observation of crossing-over in the four-strand stage in *Drosophila*. Wettstein (1924) and Allen (1928, 1930) have found "two-type" tetrads from sporophytes of *Funaria* and *Sphaerocarpos*, hybrid for supposedly linked factors. Even with Allen's suggestion that the linkage might be of the *Oenothera* type, a two-type tetrad would ordinarily mean crossing-over in the two-strand stage. The two-type tetrad could, however, be explained by the assumption that the linked factors are in different chromosomes which usually associate and disjoin to opposite poles but occasionally fail to associate and then segregate at random to give viable gametes. The cytological condition for this is found in *Rhago* (Darlington, 1929), *Campanula* (Gairdner and Darlington, 1930) and to a less extent in *Oenothera* (Kihara, 1926). In this case the observations have no bearing on crossing-over within the chromosome. This conclusion is justified by the genetical observations, which leave room for doubt as to the linkage being of the simple *Drosophila* type. It is not excluded by the cytological observations of Lorbeer (1927).

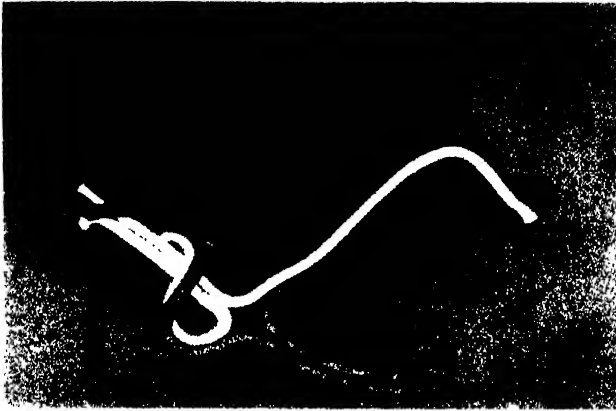


FIG. 11.—According to the assumption that no crossing over has taken place and that chiasmata are due to the opening out of successive equational and reductional splits. Certain chiasmata necessarily involve chromatids derived from more than two chromosomes.

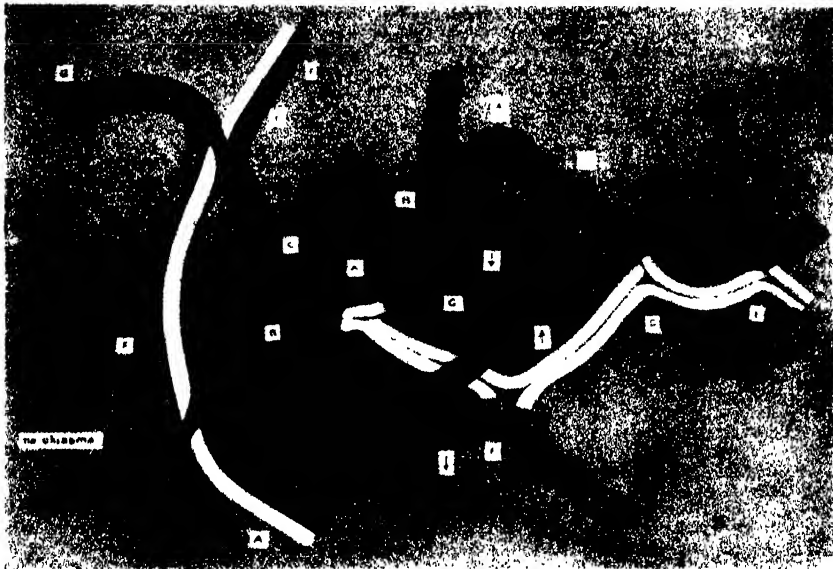


FIG. 12. The pairing of chromosomes observed at pachytene in the variety "La Grandesse." Three chromosomes never associate at any one point.

FIG. 13. The resultant configuration on the assumption that only identical threads are paired and that therefore every chiasma represents, and is the result of, a crossing-over between two chromatids. The chiasmata are lettered to show how they correspond with positions on the pachytene chromosomes (fig. 10).

FIGS. 11-13. — Photographs of models to show possible genetical interpretations of chromatid relationships at figs. 6 and 10.

(Facing p. 58.)



## REFERENCES.

- Allen, C. E. (1926a). 'Proc. Nat. Acad. Sci.,' vol. 12, p. 2.  
 Allen, C. E. (1930). 'Genetics,' vol. 15, p. 150.  
 Bateson, W., Saunders, E. R., and Punnett, R. C. (1905). 'Rep. Evol. Comm. (Roy. Soc.)' vol. 11, p. 80.  
 Bélař, K. (1928). "Die cytologischen Grundlagen der Vererbung." (Berlin.)  
 Belling, J. (1926). 'Biol. Bull.,' vol. 50, p. 355.  
 Belling, J. (1927). 'Biol. Bull.,' vol. 53, p. 480.  
 Belling, J. (1928). 'Univ. Calif. Pub. Bot.,' vol. 14, p. 283.  
 Belling, J. (1928). 'Biol. Bull.,' vol. 54, p. 465.  
 Belling, J. (1929). 'Univ. Calif. Pub. Bot.,' vol. 14, p. 379.  
 Boveri, Th. (1904). "Ergebnisse über die Konstitution der chromatischen Substanz des Zellkerns." (Jena.)  
 Chodat, R. (1925). 'Bull. Soc. Bot. Genève,' 1925.  
 Correns, C. (1902). 'Bot. Z.,' vol. 60.  
 Darlington, C. D. (1929a). 'J. Genet.,' vol. 21, p. 17.  
 Darlington, C. D. (1929b). 'J. Genet.,' vol. 21, p. 207.  
 Darlington, C. D. (1930a). 'J. Genet.,' vol. 22, p. 65.  
 Darlington, C. D. (1930b). 'J. Genet.,' vol. 22 (in press).  
 Gairdner, A. E., and Darlington, C. D. 'Nature,' vol. 125, p. 87.  
 Janssens, F. A. (1909). 'Cellule,' vol. 25, p. 387.  
 Janssens, F. A. (1924). 'Cellule,' vol. 34, p. 135.  
 Jennings, H. S. (1923). 'Genetics,' vol. 8, p. 393.  
 Kihara, H. (1927). 'Jahrb. Wiss. Bot.,' vol. 66, p. 429.  
 Lorbeer, G. (1927). 'Z. Indukt. Abstamm. Vererb. Lehre,' vol. 44, p. 1.  
 McClung, C. (1914). 'J. Morphol.,' vol. 25, p. 651.  
 Maeda, T. (1930a). 'Mem. Coll. Sci., Kyoto,' B, vol. 5, p. 89.  
 Maeda, T. (1930b). 'Mem. Coll. Sci., Kyoto,' B, vol. 5, p. 125.  
 Morgan, T. H. (1911). 'Science,' vol. 34, p. 384.  
 Newton, W. C. F. (1927). 'J. Linn. Soc.,' vol. 47, p. 339.  
 Newton, W. C. F., and Darlington, C. D. (1929). 'J. Genetic,' vol. 21, p. 1.  
 Newton, W. C. F., and Darlington, C. D. (1930). 'J. Genetic,' vol. 22, p.  
 Robertson, W. R. B. (1916). 'J. Morphol.,' vol. 27, p. 179.  
 Renner, O. (1929). "Handb. Vererbungswissenschaft."  
 Rückert, J. (1892). 'Anat. Anz.,' vol. 7, p. 107.  
 Sella, J. (1928). 'Arch. Zellf.,' vol. 16, p. 171.  
 Weirich, D. H. (1916). 'Bull. Mus. Comp. Zool. Harvard,' vol. 66, p. 57.  
 Wettstein, F. v. 'Z. Indukt. Abstamm. Vererb. Lehre,' vol. 22, p. 1.  
 Wilson, E. B. (1925). "The Cell, New York."



*The Development and Vascularisation of the Corpus Luteum in the Mouse and Rabbit.*

By RUTH DEANESLY, Mackinnon Student of the Royal Society.

(Communicated by J. P. Hill, F.R.S.—Received July, 1930.)

[PLATES 6-8.]

*Introduction.*

The development of the corpus luteum from the ruptured follicle has been the subject of a very large number of memoirs. Of these the most important have been summarised by Van der Stricht (1912) up to 1912 and more recently by Hill and Gatenby (1926) and Corner (1919), so that no useful purpose would be served by recapitulating in detail the controversial discussions contained in them. Many of the older papers are not based on a complete series of early stages of corpora lutea, since either the material was not available, or else the writers were unable to diagnose accurately the time of œstrus. Sobotta (1890, 1897), Cohn (1903), Marshall (1904, 1925), and Van der Stricht (1912), however, drew their conclusions from a series of accurately dated ovaries, as have more recent workers (Corner, 1919; Drips, 1919; Hill and Gatenby, 1926; Kurashige, 1927; Long and Evans, 1922; and Watrin, 1924).

It is now generally agreed—and has been shown conclusively in the work of Hill and Gatenby (1926)—that the lutein cells are formed by the enlargement of the follicular epithelium. The fate of the theca interna cells, which surround the mature follicle prior to ovulation, is still a matter of dispute, and the problem is complicated by histological species differences. There seems no reasonable doubt, however, that these elements take part in the formation of the corpus luteum, since, in numerous cases, the typical fat-containing cells, closely associated with the vascular connective tissue, can be distinguished among the follicular lutein cells after ovulation. It remains to be decided whether or not they persist as functional elements, and retain their individuality.

These cells, in some species, can be traced in the corpus luteum for a considerable time, since they differ in size from the lutein elements, as in the Monotreme (Hill and Gatenby, 1926) where they are much smaller, and in the human (Watrin, 1925) where they are abundant and initially larger than the lutein cells. There is evidence, however, in these cases, that the theca interna cells appear degenerate when the corpus luteum has attained full development.

In other species, such as the bat, rat, rabbit and mouse, the corpus luteum, once fully formed a few days after ovulation, appears to consist only of lutein cells of a single type with blood vessels and reticular tissue ramifying among them. Under these circumstances the transformation of the previously distinct theca cells into either lutein cells or reticular connective tissue must be assumed, unless evidence of extensive cell degeneration can be found. Van der Stricht (1912) holds that the theca interna of the follicle has incorporated "interstitial cells," which function as lutein cells in the fully formed corpus luteum, and become indistinguishable from the true lutein cells formed from the follicular epithelium. Sobotta (1896, 1897), Cohn (1903), Marshall (1904, 1905, 1925) and more recently Drips (1919) consider the theca interna cells as all belonging to the connective tissue group, and in no way secretory. Corner's views (1919) are in some respects intermediate, and have been criticised (Gatenby, Solomons and Gatenby, 1924); he apparently regards the theca interna cells as partially distinct elements which approximate to lutein rather than connective tissue cells. Watrin (1924, 1926) who has studied a very large number of human corpora lutea, including early post-ovulation stages, regards the thecal cells as nutritive and not secretory.

There is no general agreement between different writers as to the characteristics of these elusive cells, except for the fact that shortly before and after ovulation lipoids or osmicated fat may be found in their cytoplasm, when suitably fixed. Corner (1919) and Horrenberger (1928) endeavour to distinguish two types of theca interna cell. In platypus (Hill and Gatenby, 1926) the theca interna consists of small compactly arranged cells, which are not fibroblastic, but in most laboratory animals the cells are more variable and of a different appearance. Capillaries have been noted in the theca interna in platypus, mouse, ferret, bat, rabbit, pig and man, and it seems probable that they are generally more abundant in this region than in the theca externa.

Contrary to the views of Corner and Van der Stricht it should be noted that where the theca cells remain most distinct, e.g., in man (Gatenby, 1924; Solomons and Gatenby, 1924; Horrenberger, 1928; and Watrin, 1924) and platypus (Hill and Gatenby, 1926) they show no tendency to approximate to true lutein cells, their affinities being definitely with the connective tissue group. Gatenby (*loc. cit.*) and Horrenberger (1928) bring forward cytological evidence indicating that derivatives of the theca interna have a secretory function in the human corpus luteum, but Watrin as stated above, working with a more complete series of the same material, is in disagreement with these authors on this point.

From the literature, very briefly reviewed above, it may be concluded that the evidence for the formation of true lutein cells from the theca interna is altogether inconclusive and may be rejected, since it depends largely on the fact that the cell contents in the theca elements at the time of ovulation resemble histologically those found later in the lutein cells. It is very doubtful if the variations in staining recorded by Corner (1919) in the lutein cells of the fully developed sow corpus luteum, indicate the presence of two different types of lutein cell. They may with more probability be attributed to differences in the fat and lipid cell inclusions among similar cells resulting from different phases of secretory activity. Corner himself records a failure to distinguish "theca lutein" cells in either the mouse or the rat.

The problem still at issue, therefore, appears to be whether the theca interna cells eventually degenerate, give rise to the corpus luteum reticulum, or form specific secretory elements. The following account, in which a solution is attempted, is based on corpora lutea of known age from normal mice and rabbits; much of the material was fixed so as to preserve the fat and lipid cell inclusions, which were not described by Sobotta in his classic papers. For purposes of histological investigation the disadvantage of the smallness of the mouse corpus luteum is outweighed by the possibility of obtaining a large number of early developmental stages, and by the important fact that the lutein cells contain only very fine granulations, differing from the cell inclusions in the theca interna when the latter is most prominent, shortly after ovulation. In view of papers already published (Sobotta, 1897; Cohn, 1903; Kurashige, 1927, and Marshall, 1925) the rabbit corpus luteum is not described in detail. The term "lutein cells" is here restricted to the cells originally forming the follicular epithelium, while "theca interna cells" applies only to those recognisable as such by their peripheral position or cell inclusions; "fats and lipoids" refer to the cytoplasmic substances seen in sections through the corpus luteum, prepared according to one or other of the methods given below.

#### *Material and Technique.*

*Material.*—Ripe follicles and newly-formed corpora lutea were examined in 22 ovaries from 12 mice; the corresponding stage of the oestrous cycle was noted by observation of the vaginal smear, parturition or the vaginal plug (Allen, 1922; Parkes, 1926). Ovulation has been shown to take place in the mouse during late pro- or early oestrous (Brambell, 1928). For the study of older, fully developed corpora lutea numerous other mouse ovaries were available, the material described here being part of a collection of about 80 ovaries,

showing the condition of the corpora lutea in relation to the oestrous cycle (Deanesly, 1930). Three of the mice examined had ovaries containing ripe follicles; in five there were newly formed corpora lutea, in which vascularisation had only just begun, and in the other four mice, killed just before and after metoestrus, the progress of corpus luteum vascularisation and enlargement of the lutein cells could be traced. Measurements indicate that the corpus luteum of ovulation shows little or no further increase after it is 48 hours old, namely, by about the end of metoestrus.

The stages of rabbit corpus luteum development were studied in ovaries obtained 18, 24, 36, 55 hours and 5 and 8 days after copulation; ovulation takes place about 10 hours after copulation (Marshall, 1925).

*Technique.*—The histological methods have been more fully described elsewhere (Deanesly, 1930). Flemming's and Ciaccio's fluids were used as a routine for the two ovaries of each mouse, but in a few cases Bouin's fluid was used for the one ovary, the second being preserved in one or other of the above fixatives. Sections to show osmicated fats or lipoids were mounted in Farrant's glycerine medium. Lipoids were stained with Sudan III or Scharlach R. after Ciaccio fixation. Complete serial sections were cut at  $7\ \mu$  of most of the mouse ovaries. Rabbit ovaries were treated similarly, but were cut in pieces before fixation, only those containing young corpora lutea being sectioned. The same stage could be examined in pieces from a single ovary, differently fixed.

Iron hæmatoxylin and occasionally safranin was used after Flemming's and Ciaccio's fixatives, and Ehrlich's or Delafield's hæmatoxylin after Bouin. As counterstains, eosin, Van Gieson, Pasini, and Masson's Ponceau red followed by light green were used and served to show up the reticular connective tissue.

Cell size in the different corpora lutea was compared by means of a Reichert microscope fitting which enabled slides under two similar microscopes to be focussed into the same field. Direct comparison of corresponding areas of sections in different ovaries is thus possible. This is much more satisfactory than measurements of a limited number of individual cells (which was also undertaken), owing to the normal variation.

To estimate the size of corpora lutea from their appearance in section, two diameters at right angles to each other, were measured across a number of corpora lutea produced at the same ovulation; the average of these measurements was taken to represent the mean diameter of the corpus luteum at that period of the oestrous cycle.

*The Development of the Mouse Corpus Luteum.*

*The Mature Follicle.*—The growth of the Graafian follicle has been described in detail by Brambell (1928), who gives the mean diameter of the ripe follicle of the mouse, in section, as about 530  $\mu$ . In the present material, follicles ranging from 430 to 530  $\mu$  were found in three mice, killed during early pro-œstrus, œstrus and parturition. A portion of the edge of a ripe follicle is shown in fig. 1, Plate 6. The follicular cells have little cytoplasm and their nuclear diameter is 5  $\mu$ . At about the time of pro-œstrus these cells, previously fat-free in section, begin to show very fine osmicated granules and lipoids staining with Scharlach R. in their cytoplasm. These granules appear first in the peripheral cells, but later, as ovulation approaches, they extend throughout the follicular epithelium.

The theca interna in the ripe follicle consists of a layer, one or two cells deep of irregularly arranged, fat-containing cells intimately associated with congested capillaries and fibrous connective tissue. The nuclei of the theca interna cells are mostly round or oval, and similar in size to those of the follicular epithelium, but more lightly staining; their cell limits cannot often be clearly distinguished. The fat and lipid cell inclusions stain similarly to those in the stroma of the ovary; the granules are much coarser and more irregular than those in the follicular epithelium. The theca externa, surrounding the follicle, consists mainly of fibrous connective tissue, but includes some capillaries.

*Four and a half hours after Copulation.*—The youngest corpora lutea examined were those in a mouse killed 4½ hours after copulation (fig. 5). Their mean diameter is about 420  $\mu$  showing that the follicle has contracted slightly after expulsion of the ovum. All the corpora lutea were solid, but the ovulation point could still be distinguished. The follicular epithelial, or lutein cells, as they will now be termed, have not changed appreciably since ovulation, and their cytoplasm still contains fine osmicated granules. The theca interna has greatly enlarged; it forms an irregular fatty zone, four to eight cells thick, containing capillaries, and projecting inwards among the lutein cells. In sections where the fatty cell inclusions are not shown there is no clear distinction between the theca interna and externa; both appear to consist of cells of a connective tissue type and capillaries, showing indications of active growth. Fat-containing cells, however, are mainly confined to the inner zone. The average size of the characteristic theca interna cells is larger than in the mature follicle, and their light staining vesicular nuclei now exceed those of

the lutein cells in size. Not all the fatty cells, however, have nuclei of this type; some have elongated nuclei and branching cytoplasm. Although the theca interna cells appear to be pushing inwards among the lutein cells, closely associated with capillaries, they retain their connection with the sheath, and do not penetrate more than a fifth of the distance towards the centre of the corpus luteum. No detached cells of this type can be seen among the lutein elements, although mitoses are fairly common among the innermost sheath cells. There are a few spindle cells, however, of a fibroblastic type beginning to push in among the lutein cells from the sheath; these are still comparatively rare, and they are only found near the edge of the corpus luteum.

*Seven to Ten Hours after Ovulation.*—The next stage of corpus luteum development is shown in the ovaries of four mice killed at about 10 o'clock in the morning, following ovulation during the previous night (fig. 6). Comparison with the earlier stage indicates that these corpora lutea are about 3 to 6 hours older; the differences between them are slight compared to their difference from the 4½-hours stage. The lutein cells have not increased in size, but have lost much of their osmicated fat and lipoids, though faint granulations still persist in their cytoplasm. The theca interna cells (fig. 2) have not changed appreciably, and both theca interna and externa are still in a state of active growth; springing from the sheath there is now a network of spindle cells which have penetrated throughout the corpus luteum. These do not resemble the theca interna cells; instead of the coarsely granulated fat characteristic of the latter, the spindle cells contain only very fine fatty granules (fig. 3); their cytoplasm stains black with iron hæmatoxylin after Flemming fixation (fig. 2). Capillaries are still confined to the outer parts of the corpus luteum.

*About 16 Hours after Ovulation.*—Corpora lutea at this stage were obtained from a mouse killed late on the day following parturition; they are therefore 6 to 9 hours older than those just described. Their mean diameter shows no increase in size, and the lutein cells have not yet begun to enlarge. They now contain no osmicated fat granules in section. Capillaries have spread to the centre of the corpus luteum, but mitoses are still occurring in the vascular connective tissue. Fat granules can no longer be distinguished in the ingrowing fibroblastic spindle cells. The theca interna has undergone marked changes (fig. 4) and is now far less prominent; fatty cells are less abundant, and among those still persisting the nuclear variations have become accentuated. The majority of the nuclei are dark staining and very irregular, and there is an average decrease in the nuclear size in comparison with the last stage. In some cells the nuclei appear so shrunken as to be almost pycnotic, but even if

it be assumed that these nuclei are associated with degenerating cells, which is by no means certain, they are wholly insufficient in number for the death of all the theca cells visible at the earlier stages to be postulated.

*Two or Three Days after Ovulation.*—Three unmated mice were killed during early and late metoestrus, and the corpora lutea in their ovaries show only minor differences from each other. The mean diameter has increased since the last stage to  $560\ \mu$ , owing to the enlargement of the lutein cells. Their nuclei have a diameter of 6 to  $7.7\ \mu$ , while that of the cells is about  $10\ \mu$ ; the latter are variable and the cell limits not very distinct. The corpus luteum of ovulation has now reached its full size, and no further enlargement of the lutein cells takes place. Osmicated fat, in fine granules, is only found in the peripheral cells, but lipoids are again present throughout the corpus luteum, though as yet in small quantities. The theca interna had disappeared as such, and there is no longer a definite border of fatty cells, though in the thin layer of fibrous connective tissue surrounding the corpus luteum an occasional cell of this type can be found—perhaps part of the original theca interna, perhaps belonging to the ovarian stroma.

The absence at any time of cells of a specific theca interna type, distinguishable as such within the corpus luteum and detached from the actual sheath, the mitoses and changes which take place in the theca interna between ovulation and the full development of the corpus luteum, and lastly the presence of fine fat granules in the inpushing fibroblastic spindle cells 8 hours after ovulation indicate clearly that in the mouse the theca interna gives rise to a branching reticulum, and does not retain its individuality or become transformed into lutein cells.

The fibroblastic cells derived from the sheath can be traced in the fully-developed corpus luteum, since, after Flemming fixation, the affinity of their cytoplasm for iron hæmatoxylin renders them conspicuous. In metoestrous and dioestrous corpora lutea of ovulation, cells of this kind line the blood spaces, their fibres forming a network among the lutein cells. The oval nuclei are larger and more lightly staining than endothelial nuclei of adult tissues (fig. 7, Plate 7).

The corpus luteum of ovulation in the human is stated to be inadequately vascularised by comparison with that of pregnancy (Watrin, 1924); in the mouse the same contrast can be observed if the corpus luteum of ovulation be compared with that of either pregnancy or pseudo-pregnancy. In the latter the cells forming the fibrous reticulum are more numerous and their nuclei are smaller and more darkly staining than in the corpus of ovulation; as before,

however, the fibrous cells line the blood channels permeating the corpus luteum (fig. 8). The transition from the irregular fibroblastic tissue in the corpus luteum of ovulation to the vascular network of the fully-developed organ can be traced by comparing a mouse corpus luteum, 3 to 4 days after sterile copulation, showing the intermediate condition, with fully-grown corpora lutea of ovulation, on the one hand, and pseudo-pregnancy or pregnancy, on the other.

Corner after studying the corpus luteum of the sow also concluded that the fibrous reticulum was derived from the endothelial cells (Corner, 1919), and subsequently, using the Bielschowsky Maresch technique, confirmed his conclusion (Corner, 1920).

In view of the continuity which can be traced between the fibres of the sheath, and those within the fully-formed organ, it seems probable that the latter are closely related to ordinary collagenous connective tissue such as can be seen forming septa in the corpora lutea of larger mammals. The affinities of the corpus luteum vascular connective tissue are further discussed below.

#### *The Development of the Rabbit Corpus Luteum.*

*The Mature Follicle.*—The ripe follicle of the rabbit was examined only in Bouin fixed material. The liquid folliculi occupies a larger proportion of the volume of the follicle than it does in the mouse. The nuclei of the follicular cells have a diameter of about  $7\ \mu$ , that of the cells being about 10 to  $11\ \mu$ . A single layer of theca interna cells can be distinguished; they have more cytoplasm than the epithelial cells, and their oval light staining nuclei are also larger, measuring about  $9\ \mu \times 6.7\ \mu$  in diameter. The absence of cytoplasmic vacuoles, in the material examined, indicates a lack of the coarse fat which occurs at this stage in the corresponding cells of the mouse. On both sides of this layer of theca interna cells lie capillaries, which become congested prior to ovulation. The theca externa includes further capillaries and fibrous connective tissue.

*Corpus Luteum 18 Hours after Copulation.*—The ovaries from two rabbits were examined, fixed in Flemming's, Ciaccio's and Bouin's fluids. Since ovulation occurs about 10 hours after copulation, the corpora lutea were about 8 hours old. After expulsion of the egg the follicle contracts to about half its previous size in section, as the result of which both theca interna and externa increase in thickness. The appearance of the young corpus luteum at this stage is shown in figs. 9 and 10 (Plate 8). Neither epithelial nor theca interna cells have increased in size since ovulation, but mitoses are occurring among the latter, and both theca interna and externa appear in an actively



growing state. Elongated, spindle-shaped cells, springing from the sheath, have penetrated to the central cavity of the corpus luteum, into which an effusion of red blood corpuscles has taken place (*cf.* Marshall, 1925). The spindle cells resemble those found in the mouse corpus luteum, at the corresponding stage of development; after Flemming fixation, their cytoplasm stains black with iron hæmatoxylin (fig. 9).

The corpus luteum as a whole contains little stainable fat or lipoids; with Scharlach R. after Ciaccio fixation, it is pale orange in section, and after Flemming fixation, only traces of fine osmicated granules can be found. Under high magnification, the fat and lipid granules are seen in the lutein cells, in the theca interna cells, and in the spindle cells springing from the sheath. Thus their distribution is the same as in the mouse corpus luteum at the same stage of development; the fat in the theca interna cells of the rabbit, however, consists of fine granules, similar to those in the lutein cells, and is much less conspicuous than that in the corpus luteum of the mouse (fig. 5, Plate 7).

*The Corpus Luteum 24 Hours after Copulation.*—At this stage there appears to be some increase in the fat granulation throughout the corpus luteum, but it is still very slight; the increase is most apparent in the cells of the theca interna. It is during this period that the zone reaches its maximum development; the characteristic fat-containing cells with large vesicular nuclei have increased in number and size within the last 6 hours (figs. 10 and 11) and some are in mitosis. Capillaries and fibroblastic cells ramify among them, and lie between them and the lutein cells. Examination of unstained sections of Flemming-fixed material under high magnification shows that fat granules are not confined to the large cells, but occur as well in the smaller fibroblastic cells lying among them, and also in the spindle cells growing from the theca. Cells of this type, nearer the centre of the corpus luteum, such as could be seen in the last stage, have mostly lost the osmicated fat granules which they then had, but their cytoplasm still stains black with iron hæmatoxylin after Flemming fixation. Some of these cells can be seen forming the walls of capillaries, but the vascularisation is not yet regularly developed. Some capillaries are growing in from the theca externa.

The lutein cells have increased in size; the oval nuclei measure about  $8.5 \mu \times 6.5 \mu$ , and the cell diameters range from 10 to  $15 \mu$ . They are still smaller than the large cells of the theca interna. None of the latter can be distinguished lying free among the lutein cells, apart from the sheath, but owing to the similar fat granulation, their identification would be more uncertain than that of the same cell in the mouse.

*The Corpus Luteum 36 Hours after Copulation.*—The corpus luteum at this time still contains a large cavity and remains open to the exterior. The theca interna has become reduced and there is no longer a complete sheath of fatty cells. Those which persist are of a mixed type as before; there is a slight decrease in their average size, but it is not as yet so apparent as that observed in the corresponding cells of the mouse (fig. 4). Fewer theca interna cells with large vesicular nuclei can be seen, but some of these are still in mitosis. Fibroblastic cells, also containing osmicated fat granules, can be traced among them, and similar cells can be seen pushing in from the sheath among the lutein elements. No degenerating cells can be distinguished in the region occupied by the theca interna 12 hours previously.

Fig. 11 shows the spindle-shaped cells, which constitute a prominent network throughout the corpus luteum. As in earlier stages they can be seen forming the walls of blood vessels; endothelial cells with small nuclei, such as line the capillaries of fully-developed corpora lutea, are very rare. The large fibroblastic cells constitute both the walls of the blood vessels as well as the supporting tissue of the corpus luteum.

In comparison with the last stage, a decrease has taken place in the fat granulation of the lutein cells, such as occurs in the mouse corpus luteum when it is first vascularised, and the corpus luteum is now practically fat free. The lutein cells show a considerable increase in size, and measure about  $17 \times 10 \mu$  (fig. 11).

*The Corpus Luteum 55 Hours after Copulation.*—This corpus luteum is no longer open to the exterior, but there is still a central cavity. The theca interna cannot be distinguished between the fibrous theca externa and the lutein cells. The latter have increased in size, and the diameter of the cells ranges from  $16 \mu$  to  $32 \mu$ , while that of the nuclei is  $8 \mu$  to  $9 \mu$ . These cells appear almost fat free, but in some fine osmicated granules can be seen.

Apart from the larger size of the lutein cells, the chief difference between this corpus luteum and the last one examined lies in the vascular connective tissue, which has undergone considerable proliferation. The large fibroblastic cells bordering the capillaries have given place to others, more numerous, with nuclei of the small, darkly-staining endothelial type. Some of the nuclei are transitional in size and some are still in mitosis. The change is exactly similar to that which occurs in the developing corpus luteum of pseudo-pregnancy of the mouse, and is even more readily traced in the rabbit.

*The Corpus Luteum at 4 Days 19 Hours after Fertile Copulation.*—The corpus luteum is now solid, and the lutein cells have reached their maximum size;

the nuclei are still  $8\ \mu$  to  $9\ \mu$  in diameter, but the cells measure  $20\ \mu$  to  $38\ \mu$ . They contain osmicated fat granules in considerable quantity, and similar granules occur in some of the reticulo-endothelial cells. The nuclei of the latter show a further slight reduction in average size. The fibrous endothelial tissue is very abundant and ramifies round every lutein cell. No second type of lutein cell, such as has been described by Corner (1919), can be distinguished in the fully-developed corpus luteum, with the histological methods used.

It is clear from the above description that study of the rabbit corpus luteum, by the same three methods used for the mouse, reveals an essentially similar process of development. The salient points common to both animals, concerning the fate of the theca interna may be summarised as follows :—

- (1) The theca interna at the time when it is most prominent, includes cells with large vesicular nuclei and others of a fibroblastic type, both kinds containing fatty cytoplasmic granules.
- (2) Fatty granules also occur in the fibroblastic spindle cells which grow inwards from the sheath, and spread throughout the corpus luteum ; the part played by these cells in the establishment of the vascular system can be clearly traced.
- (3) During the vascularisation of the corpus luteum, the theca interna, though including cells in mitosis, becomes reduced and disappears. No degenerating cells can be found in the region formerly occupied by it in the rabbit, and only a few doubtful ones in the mouse.

It follows that the presence of fat and lipid granules in the cells of the theca interna, which show great variability, cannot by itself establish them as specific secretory elements ; on the other hand, the cell divisions in the sheath and its gradual disappearance indicate the part it plays in the vascularisation of the developing corpus luteum.

Owing to the similarity of the fine fat granulation in the theca and in the lutein cells, in the early stages, direct immigration of the former cells among the latter cannot be excluded in the rabbit as definitely as it can in the mouse. At the same time, however, there appears to be always a border of fibrous cells between the lutein and the large theca interna elements (figs. 10 and 11).

The developmental process summarised above corresponds with that described by Cohn (1903), who does not record, however, the fatty granules in the fibroblastic cells invading the corpus luteum from the sheath. (These granules are very readily dissolved out by xylol, or Canada balsam in xylol,

and can only be observed in sections mounted in a glycerine medium, or examined immediately after mounting in balsam.)

Kurashige (1927) also concludes that the theca interna in the rabbit forms the reticulum of the corpus luteum.

#### *Discussion.*

There seems no reason to believe that the development and adult structure of the corpus luteum of the mouse and rabbit is radically different from that of other mammals, though possibly Monotremes may show a divergence. Marshall's description of the corpus luteum of the sheep (1904), Corner's of the sow (1919), Sobotta's (1897), Cohn's (1903), Kurashige's (1927), and Marshall's (1925) accounts of the rabbit, all compiled from accurately dated material, describe in slightly different terms what appears to be the same process of ingrowth and multiplication of fibrous cells from the theca. Most authors refer to variations in the characteristics of the theca interna cells, and to the irregular manner in which the vascularisation is established (Cohn, 1905; Corner, 1920; Watrin, 1926; and Marshall, 1925). In all probability the chief differences between the developing corpora lutea of some mammals and others can be attributed to the great variation in the size of the ripe follicles. In man and some of the larger animals the newly formed corpus luteum consists of a thin layer of follicular epithelium surrounded by a theca which, since the discharge of the ovum and the liquor folliculi, has become relatively thick and thrown into folds. In such corpora lutea the theca interna cells are very numerous in comparison with the lutein cells and are only slowly utilised as vascularisation proceeds; numbers of them may persist unchanged for a considerable time, though eventually they always degenerate or disappear, Watrin's descriptions of the corpus luteum of ovulation and pregnancy in the human (Watrin, 1924) are very relevant in this connection. He notes the persistence at the periphery and along the connective tissue septa, of lipoid-containing thecal cells in a human corpus luteum of ovulation, after the vascularisation is complete, but these cells degenerate in similar corpora lutea at a later stage. In the corpus luteum of gestation, on the other hand, no thecal cells persist, when it is a month old; by comparison with this, the corpus luteum of ovulation, which has only a short functional life, appears inadequately vascularised.

In the mouse and rabbit the theca interna is rapidly used up and by 36 hours after ovulation there is generally little or no trace of it left. One mouse, 4½ days after parturition, however, had corpora lutea of lactation in which

the theca interna had persisted ; these, however, were small for their presumed age (about 3 days), the lutein cells not having enlarged, and were not fully vascularised. Presumably their development was not abnormal, but retarded.

In the absence of a full and accurately dated series of ovaries, Gatenby's preparations of corpora lutea by various special methods (Gatenby, 1924 : Solomons and Gatenby, 1924) cannot be regarded as convincing evidence of the fate of the theca interna cells. It may be suggested that the stellate cells, which blacken in silver preparations, are not specific in nature but part of the reticulum. In the mouse and rabbit the fibrous endothelial cells take up fatty substances from the lutein cells, so that under certain conditions they might resemble the secretory cells described by Gatenby. The latter are described as staining intensely with iron hæmatoxylin after ordinary fixation and containing granules which blacken with osmic acid (Solomons and Gatenby, 1924).

As regards the nomenclature of the vascular connective tissue of the corpus luteum, the term reticulo-endothelium, which would express the anatomical relations of the cells, is perhaps best avoided, owing to its physiological connotations. Maximow (1928) associates fibroblasts and ordinary endothelium together, in the same connective tissue group, while he regards reticulo-endothelium as part of the histiocyte group, on account of its phagocytic properties. It would seem rather doubtful, however, whether this is really a fundamental distinction, since Aschoff (1924) and others have shown that the phagocytic activity of the reticulo-endothelial tissues varies in different organs (Sachs, 1926). In the adrenal, for example, the reticulo-endothelial cells—which Aschoff (1924) and Maximow (1928) include as histiocytes—are comparatively inactive in storing vital stains (Cappell, 1929). The corresponding cells of the mouse corpus luteum, which are anatomically similar, show even less affinity for vital stains, though occasional cells will take up trypan blue.\* They are active, however, in taking up fatty substances from the lutein cells, and Cappell states, with reference to lipoid containing cells in the testis (p. 601), "cells loaded with a large amount of one substance, as a rule, fail to take up intensely another injected subsequently."

Associated with the problem of the nature of the vascular connective tissue of the corpus luteum is that of the theca interna whence it is derived. Cappell and Goldmann (1929) find that the theca interna cells may contain vitally stained granules in maturing follicles, but show a different type of granulation from true histiocytes. It seems possible, however, that

\* Unpublished work by the writer.

theca interna cells are akin to histiocytes, since, according to Maximow (1928) the latter, especially in the rabbit, frequently contain fat or lipoid granules; such granules occur both in the theca of the ripe follicle and in the spindle cells which spring from it soon after ovulation, and ultimately give rise to the vascular connective tissue network. It may be concluded that the latter shows affinities both to the reticulo-endothelial or histiocyte group, on the one hand, and to ordinary endothelium and fibrous tissue on the other. Maximow, Sachs and Cappell all agree that ultimately these two main groups cannot be completely separated since intermediate forms can be found.

Looper and Looper (1930) in a note on argyrophile fibres in the rat ovary state that they form the membrana propria in the theca folliculi, while fibres continuous with this constitute the early stroma of the corpus luteum; these authors regard the argyrophile (reticular) fibres as precursors of true collagenous fibres, which increase as the corpus luteum ages, but as not identical with them. There seems no doubt that the reticular fibres of the endothelial cells are closely related to ordinary collagenous connective tissue, such as can be found in the septa of larger types of corpora lutea.

#### *Summary.*

A description is given of the development of the corpus luteum in the mouse and the rabbit, up till the time when the lutein cells, formed by the enlargement of the follicular epithelium, have reached their full size. The theca interna gives rise to the vascular connective tissue of the corpus luteum; at its maximum state of development it includes numerous fat-containing cells, some of a fibroblastic type, and others with large oval vesicular nuclei. These cells do not become detached from the sheath but divide mitotically shortly after ovulation and give rise to fibroblastic cells containing fine cytoplasmic fat granules, which penetrate inwards, and constitute both the walls of the blood spaces and the supporting tissue of the corpus luteum. The repeated division of these cells produces a network of endothelial cells, with small darkly-staining nuclei, whose fibres ramifying among the lutein cells constitute the corpus luteum reticulum. During the vascularisation the large cells of the theca interna gradually disappear, leaving no evidence of wholesale degeneration; 36 hours after ovulation little or no trace of the theca interna persists. The theca externa takes part in the vascularisation and forms the sheath of the fully-grown corpus luteum, but the more active growth comes from the theca interna. It is suggested that the cells of the latter show affinities to the histiocytes of connective tissue.

The conditions in the developing corpus luteum of the mouse and rabbit are contrasted with those described by other writers in man and larger mammals, where the theca interna cells are more abundant in proportion to the lutein elements.

The work was done in the Department of Anatomy and Embryology, University College, London.

I wish to thank Prof. J. P. Hill, F.R.S., and Dr. H. M. Carleton for reading the paper, and Dr. A. S. Parkes for supplying the material.

The histological expenses of the work were defrayed by a grant from the University of London Dixon Fund.

#### DESCRIPTION OF PLATES 6-8.

*Abbreviations.*—*b.c.*, red blood corpuscles; *cap.*, capillary; *f.*, fat; *f.e.*, follicular epithelium; *fib.*, fibroblastic cell; *l.c.*, lutein cell; *t.ext.*, theca externa; *t.int.*, theca interna cell; *m.*, mitosis; *v.c.t.*, vascular connective tissue.

*Note.*—With the exception of the last three figures, the microphotographs show sections of material fixed in Flemming; figs. 10 to 12 are taken from material fixed in Bouin. The sections shown were stained in iron hæmatoxylin, or unstained and mounted in Farant's medium.

#### PLATE 6.

- FIG. 1.—Part of the edge of a ripe follicle of a mouse, showing follicular epithelium, theca interna and theca externa.  $\times 720$ .
- FIG. 2.—Part of the edge of a developing mouse corpus luteum about 8 hours after ovulation. The dark staining fibroblastic spindle cells are pushing in among the lutein cells from the much enlarged theca interna.  $\times 720$ .
- FIG. 3.—The same stage as fig. 2; a photograph of an unstained section showing the heavy fat in the theca interna, and fine fat granules in the lutein cells and in the impushing fibroblastic spindle cells (see also fig. 6).  $\times 720$ .
- FIG. 4.—Part of the edge of a developing mouse corpus luteum about 16 hours after ovulation. In comparison with fig. 3 this shows a marked decrease in the size of the theca interna cells, and variations in their nuclei.  $\times 720$ .

#### PLATE 7.

- FIG. 5.—Unstained section through a mouse corpus luteum,  $4\frac{1}{2}$  hours after copulation, showing osmicated fat in the theca interna and in the lutein cells. The corpus luteum is already solid.  $\times 168$ .
- FIG. 6.—Unstained section through a mouse corpus luteum about 8 hours after ovulation, showing in comparison with fig. 5, a decrease in the fat granulation of the lutein cells. Active inward growth from the theca interna is now taking place (cf. figs. 2 and 3).  $\times 168$ .



f.e. t.int. t.ext.



l.c.

fib.

t.int. t.ext.



fib.

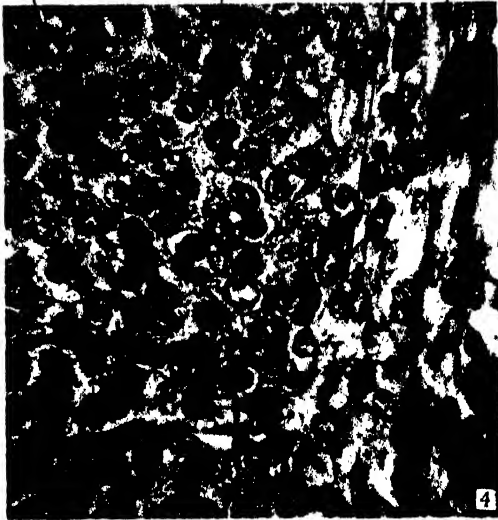
t.ext.

fib.

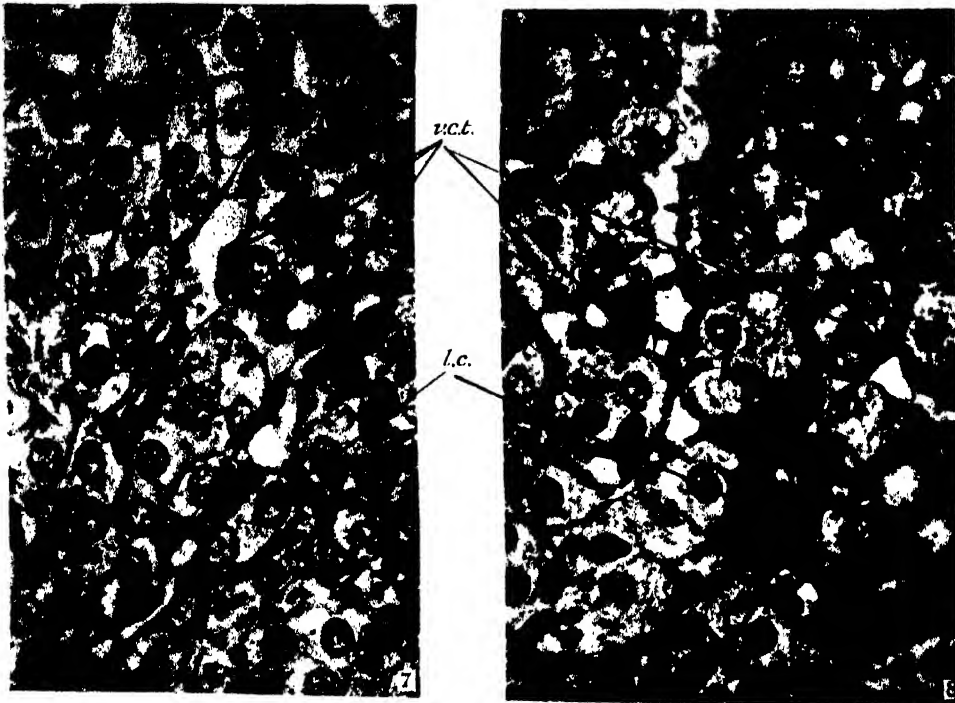
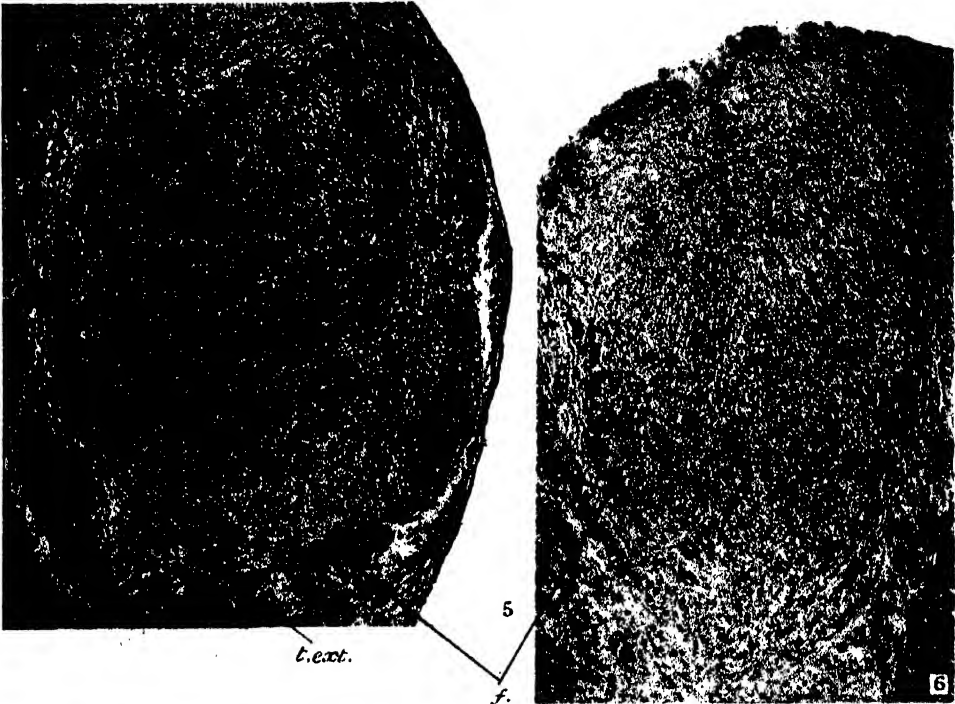
l.c.

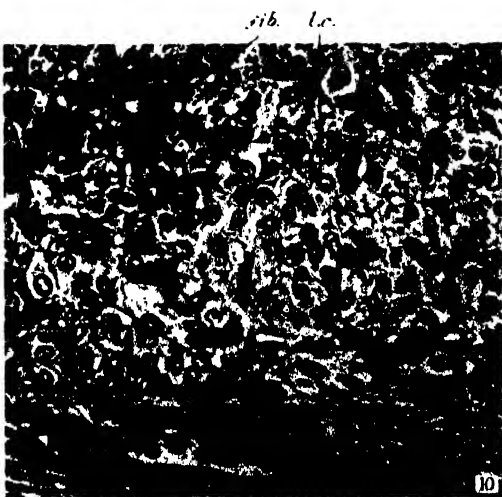
t.int. t.ext.

l.c. t.int.









t. ext.



fib.  
t. int.



t. int

t. ext

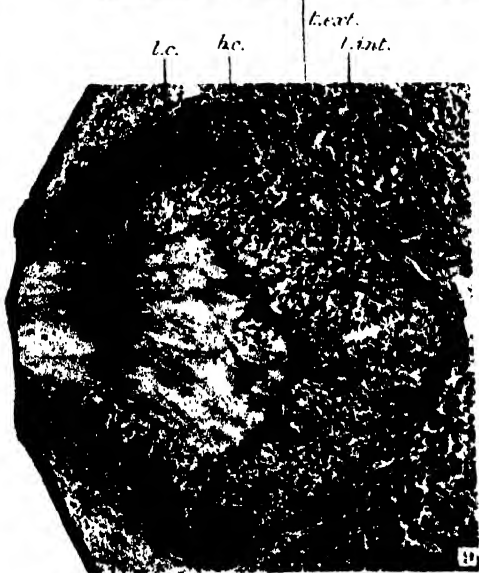




FIG. 7.—Part of a section through a fully-developed corpus luteum of ovulation of a mouse showing the large nuclei of the fibroblastic cells lining the capillaries.  $\times 720$ .

FIG. 8.—Part of a section through a fully developed corpus luteum of pseudo-pregnancy of a mouse, for comparison with fig. 7. This shows vascular connective tissue, with smaller, darker staining nuclei ramifying among the lutein cells; the latter have smaller nuclei than those in the corpus luteum of ovulation, though the cells are larger.  $\times 720$ .

PLATE 8.

FIG. 9.—Corpus luteum of a rabbit 18 hours after copulation, showing the dark staining fibrous tissue springing from the theca interna; an effusion of blood into the central cavity has taken place.  $\times 49$ .

FIG. 10.—Part of the edge of a rabbit corpus luteum, 18 hours after copulation, showing small and large cells in the theca interna, and fibroblastic tissue among the lutein cells. (Bouin.)  $\times 312$ .

FIG. 11.—Part of the edge of a rabbit corpus luteum 24 hours after copulation. The lutein cells have enlarged; some of the theca interna cells are larger than at the last stage, but between these and the lutein cells is an ingrowing border of fibrous tissue, whence spindle cells are pushing into the corpus luteum. The theca externa is also sending projections inwards. (Bouin.)  $\times 312$ .

FIG. 12.—Part of the edge of a rabbit corpus luteum 36 hours after copulation, showing much enlarged lutein cells and a greatly reduced theca interna distinguishable in section by the lighter staining cytoplasm of its cells. The spindle cells, of which one is seen in mitosis, form a prominent network. (Bouin.)  $\times 312$ .

REFERENCES.

- Allen (1922). 'Amer. J. Anat.,' vol. 30, pp. 297-348.  
 Aschoff (1924). "Lectures on Pathology." Reticulo-Endothelial System.  
 Brambell (1928). 'Proc. Roy. Soc.,' B, vol. 103, p. 258.  
 Cappell (1929). 'J. Path. Bact.,' vol. 32, pp. 595-707.  
 Cohn (1903). 'Arch. Mikr. Anat.,' vol. 62, pp. 745-772.  
 Corner (1919). 'Amer. J. Anat.,' vol. 26, pp. 117-183.  
 Corner (1920). 'Carn. Cont. to Embryology,' No. 29, pp. 87-93.  
 Deansely (1930). 'Proc. Roy. Soc.,' B, vol. 106.  
 Drips (1919). 'Amer. J. Anat.,' vol. 25, pp. 117-184.  
 Gatenby (1924). 'Proc. Roy. Irish Acad.,' B, vol. 36.  
 Hill and Gatenby (1926). 'Proc. Zool. Soc.,' pp. 715-763.  
 Horrenberger (1928). 'Arch. Anat. Hist. Emb.,' vol. 8, pp. 129-221.  
 Kurashige (1927). 'J. Coll. Agric.' (Hokkaido Imperial Univ.), vol. 20.  
 Long and Evans (1922). 'Mem. Univ. Calif.,' vol. 6.  
 Looper and Looper (1930). 'Anat. Rec.,' vol. 45, p. 229.  
 Marshall (1904). 'Phil. Trans. Roy. Soc.,' B, vol. 194, pp. 47-97.  
 Marshall (1905). 'Quart. J. Mikr. Sci.,' vol. 49, pp. 189-202.  
 Marshall (1928). "Reproduction in the Rabbit," by J. Hammond, Chap. IV, pp. 55-63.  
 Maximow (1928). "Special Cytology," vol. 1, section 14.  
 Parkes (1926). 'Proc. Roy. Soc.,' B, vol. 190, pp. 151-170.

- Sachs (1926). 'Phys. Rev.,' vol. 6.  
Sobotta (1896). 'Arch. Mikr. Anat.,' vol. 47, pp. 261-308.  
Sobotta (1897). 'Anat. Hefte,' vol. 8, pp. 469-524.  
Solomons and Gatenby (1924). 'J. Obstet. Gynec.,' vol. 31.  
Togari (1923). 'Aichi J. Exp. Med.,' vol. 1, pp. 1-44.  
Van der Stricht (1912). 'Arch. Biol.,' vol. 27, pp. 585-720.  
Watrin (1924). 'Arch. Méd. Exp.,' vol. 1, pp. 97-272.  
Watrin (1926). 'Arch. Méd. Exp.,' vol. 2, pp. 203-212.

547 . 926 . 2 : 541 . 141

### *The Distillation of Vitamin D.*

By F. A. ASKEW, R. B. BOURDILLON, H. M. BRUCE, R. G. C. JENKINS, and  
T. A. WEBSTER, National Institute for Medical Research.

(Communicated by H. H. Dale, Sec. R.S.—Received July 29, 1930.)

The work described below has been done in an attempt to isolate vitamin D from the complex mixture formed by the ultra-violet irradiation of ergosterol. It is evident that vacuum distillation might be of use for this purpose, but, except for a brief statement by Windaus and Holtz (1927), we are unaware of previous work on the distillation of vitamin D.

We have distilled various products obtained from ergosterol, and most frequently have used resinous products prepared as follows. A solution of ergosterol in ether was exposed to the unfiltered radiation from a mercury vapour lamp while flowing through a narrow silica tube at a rate such that about 40 per cent. of the ergosterol was destroyed. The unchanged ergosterol was then removed by precipitation with digitonin as described in a previous paper (Webster and Bourdillon, 1928) and the ethereal solution of the products of radiation was evaporated *in vacuo* to a dry resin.

#### *Construction of Still.*

After a number of preliminary trials, the following apparatus was assembled and used for most of our distillations. The still consisted of a straight glass tube about  $1\frac{1}{2}$  metres long and either 35 mm. or 55 mm. in diameter. This was supported in a horizontal position when in use. One end was sealed off, and was called the "boiler" end. The other was closed by a rubber cork, and connected through a tap and a short connecting tube to a two-stage Gaede mercury vapour pump, with a rotary oil backing pump.

The "boiler" consisted of a shallow glass dish placed inside the still near the sealed end. This dish was partly filled, either with the dry powdered resin, or with a concentrated solution of the resin in ether. The still was heated (after evacuation) by sliding over the glass tube an electric furnace made by winding nichrome wire round an iron pipe covered with asbestos paper. The furnace was wound so as to give a fairly uniform temperature for about 15 cm. at the "boiler" end, and then a steady fall of about  $0.4^{\circ}$  per centimetre along the tube towards the cork. A rough measure of the temperature of the still was obtained by thermometers placed at intervals of 10 cm. along the still. These passed through holes in the furnace wall, so that the stems projected outside, while the bulbs were approximately in contact with the wall of the glass still.

#### *Conditions of Distillation.*

In the majority of our experiments the temperature of the "boiler" has been approximately  $145^{\circ}\text{C.}$ , and the temperature of the condensing surface has fallen from  $145^{\circ}\text{C.}$  at the boiler end to  $95^{\circ}\text{C.}$  at the cool end of the furnace. Beyond this there was an abrupt fall of temperature to about  $18^{\circ}\text{C.}$  The time of distillation varied from 1 hour to 3 hours.

The pressure was estimated by observation of a discharge tube, with circular aluminium electrodes 2.5 cm. diameter and 8.5 cm. apart, connected to a small induction coil capable of giving a 22 mm. spark in air at ordinary pressure. The discharge tube was fused on to the glass connecting tube between the pump and the tap. During the early stages of distillation small quantities of a non-condensing vapour were evolved, and the discharge tube showed the typical green fluorescence of "an X-ray vacuum." As distillation proceeded the pressure fell until practically no discharge was visible. The pressure was then probably between 0.01 and 0.002 mm. Hg in the discharge tube. The pressure in the still must have been somewhat higher than this, owing to the obstruction produced by the tap and connecting tube, which were only of 10-mm. bore.

#### *Collection of Distillate.*

About 1 gram of resin was placed in the boiler, and of this 20 to 60 per cent. distilled, and collected as oily droplets, which adhered to the walls of the condensing tube, and solidified on cooling. In the small (35 mm.) stills the distillate was allowed to condense on the walls of the still tube itself. After distillation this tube was cut up into lengths, each containing one fraction of the distillate. The adhering resin was washed off these lengths of tube with

dry ether into small flasks, and the solutions were then evaporated *in vacuo* to dry resins, which were weighed, and redissolved in alcohol for measurement of absorption and antirachitic activity.

In the larger stills (55 mm.) a series of thin glass lining tubes, each 7.5 cm. long, were placed along the whole length of the still from boiler to cork. The distillate collected in these lining tubes, and could thus be separated into fractions. Each fraction was then dissolved and weighed as described above.

#### *Measurement of Absorption Spectra.*

The absorption spectra of the products of distillation were measured in alcoholic solution by a photographic method using a hydrogen tube as source of light, a Hilger logarithmic sector, and a microphotometer as described in a previous paper (Bourdillon *et al.*, 1930). The extinction coefficients plotted in the diagrams on this paper are calculated by the formula

$$\epsilon = (\log_{10} I_0 - \log_{10} I_1 - K)/Cd,$$

where  $I_0$  and  $I_1$  are the intensities of light before and after traversing the solution,  $C$  is concentration in grams per litre, and  $d$  is the thickness of the solution in centimetres.  $K$  is the figure representing the absorption of the silica cell when filled with solvent only. This figure is subtracted from the total absorption in order to give the absorption due to the dissolved substances only.

#### *Measurement of Antirachitic Activity.*

The measurements of antirachitic activity were carried out by the technique described in a previous paper (Bourdillon *et al.*, 1929) based on the radiographic estimation of the degree of healing of rachitic rats. The activity of each substance under test was compared with the activity of a standard solution of irradiated ergosterol kept in olive oil at 0° C. The results are expressed as percentages of the antirachitic activity of this solution, which is taken as 100.

#### *Preparation of Crystalline Product.*

On three occasions we have obtained a crystalline substance by the following procedure. One of the more volatile fractions of a distillate was re-distilled by the process described above, and one fraction of the second distillate was dissolved in alcohol to form a 0.2 per cent. solution. Water was added until the solution was opalescent, and the mixture was then allowed to evaporate slowly in a vacuum desiccator. In each case well defined crystals were deposited at an

early stage of the evaporation. These were separated by decantation, and drained on a porous plate. One specimen (D16.32) recrystallised from alcohol and water, and one (D51.2) acetone and water. Further evaporation of the mother liquors caused the deposition of liquid oils only. The crystals form clusters of rather thick needles of characteristic appearance, as shown in fig. 1, and show conspicuous double refraction in polarised light.



FIG. 1.—Crystals showing high antirachitic activity (D37.4).

These crystals have in each case shown high antirachitic activity. The values of this are shown in Table I together with the activities of the fractions from which the crystals were deposited, and other relevant data.

The numbers of rats on which each test is based are given in brackets after

Table I.

Experiment No.	Weight.	Crystals.		Fraction of distillate from which crystals were deposited.	
		Melting point.	Antirachitic activity.	Weight.	Antirachitic activity.
D.16.32	mg. 3.6	° C. 92-100	162 (8 pairs rats)	mg. 27.5	130 (3 pairs rats)
D37.4	1.5	113-115	187 (14 pairs rats)	20	70 (8 pairs rats)
D.5.12	about 9.65	—	107 (5 pairs rats)	27	160 (8 pairs rats)



each figure. Thus eight pairs of rats means that in this test eight rats dosed with the test solution showed degrees of healing that were neither too high nor too low for reliable assessment, and that each of these eight was compared with a litter mate dosed with the standard solution. We have made estimates of the standard deviation of our biological tests by a method which will be published shortly, and we conclude that the observations based on eight or more pairs of rats are reasonably accurate, so that the apparent differences between the activities of the three crystalline specimens are very probably true. Observations on two or three pairs of rats are much less reliable, and the results for the distillate fractions in Table 1 must be used with caution. The third crystalline specimen (D51.2) was very small in quantity, and was visibly contaminated with small particles of other material, which may have accounted for its low activity. D37.4 was the cleanest of the three specimens.

#### *Absorption Spectra of Crystals.*

The absorption spectra of these crystals are shown in fig. 2, together with a dotted curve, which is a copy (after recalculation in our units) of that attributed to vitamin D by Reerink and Van Wijk (1929). There is a strong similarity between all four curves, and it will be seen that the cleanest specimen of crystals showed the highest absorption as well as the highest antirachitic activity, while the most obviously contaminated specimen gave the lowest values for both properties.

A specimen of D37.4 was kept in a vacuum desiccator for 26 days, and then re-examined. It showed a fall in absorption of approximately 23 per cent., and an apparent fall in antirachitic activity of about 30 per cent., in a test based on four pairs of rats.

#### *Relation of Crystalline Product to Vitamin D.*

If there is only one substance possessing intense antirachitic activity, i.e., only one vitamin D, it is not very probable that these crystals are this substance. We have frequently obtained resinous mixtures of approximately the same antirachitic activity, although their low melting points and varying absorption spectra gave reason to believe that they were mixtures of a number of substances. Further, we have on several occasions obtained mixtures which appeared to have slightly higher activity, although these values may possibly be due to errors of testing. There are four alternative possibilities:—

1. The crystals may be an inactive substance contaminated by traces of an intensely active oil deposited on their surfaces. We think this not

very probable because the cleanest specimen obtained (D37.4) showed the highest activity and appeared free from oil. If an oil film was the source of the activity, it must have had an activity of a higher order than any that we have yet observed.

40

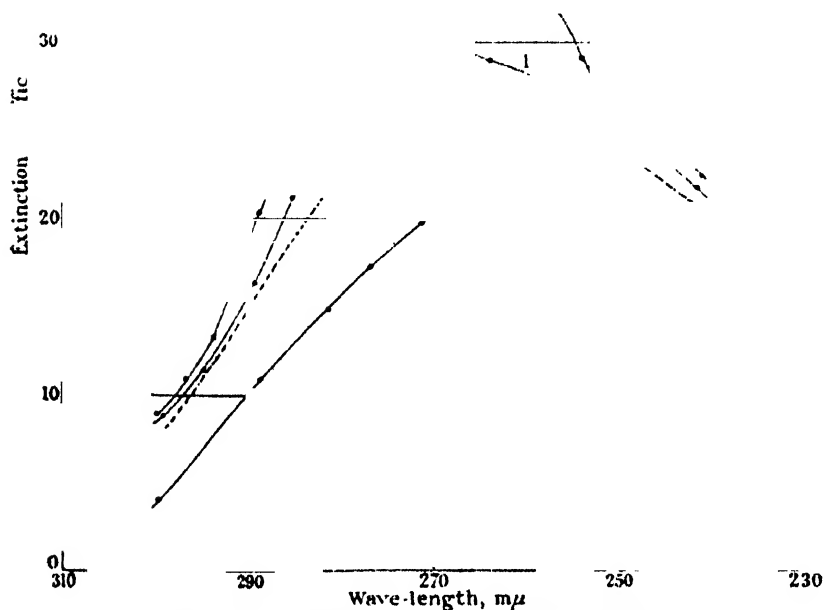


FIG. 2.—Absorption Spectra of the Crystalline Product.

No.		Antirachitic activity.
1	D16.3.2 .....	162
2	D37.4 .....	187
3	D51.2 .....	107

Curve 4 is plotted from the values attributed by Reerink and Van Wijk to pure vitamin D.

- The crystals may be a mixture in any proportion of two or more substances of sufficiently similar molecular dimensions to form homogeneous crystals, only one of them being vitamin D. This seems to us possible in view of the frequent occurrence of such mixed crystals among the sterols.
- The crystals may be of a loose compound between an active and inactive substance.

4. There may be a number of radiation products of ergosterol all possessing high but unequal antirachitic activities, i.e., a number of compounds of which any one could be called "vitamin D," but would cause only a fraction of the total activity of the ordinary irradiation products of ergosterol. This seems not improbable, and would account for the frequency with which we obtain complex mixtures of activity between 100 and 200, in contrast to the rarity with which we obtain higher values than 200.

#### *Effects of Distillation on Vitamin D.*

In 10 experiments we have measured the antirachitic activity of every fraction of the distillate, as well as the activities of the residue left in the boiler and of the original substance, and have thus obtained a measure of the destruction of vitamin during distillation. The results of these experiments are shown in Table II. Owing to the errors of biological estimation, it is unsafe to draw detailed conclusions from any one experiment, but the following deductions seem permissible.

In groups 1 and 2 there is an average destruction of about 50 per cent. of the total vitamin, as the result of an exposure of  $1\frac{1}{2}$  to 3 hours to temperatures of 100 to 145. At the same time the process usually gives at least one fraction of higher vitamin content than the original substance. In group 3 the destruction of vitamin is much greater, and the activity of the most active fraction is much less, although in one case the temperature was only 100° C. Owing to the large number of variables which can affect these results, we cannot be certain of the cause of this increased destruction. However, we suspect that it is the different technique employed for this group, namely the careful exclusion of air from contact with the preparation before distillation (*cf.* p. 89). We have done about 50 distillations in all, getting in most cases results similar to those shown in groups 1 and 2 of this table. In three further cases, however, the destruction of vitamin appeared exceptionally large. These were distillations of preparations treated as usual, except that a light filter cutting off radiations of wave-length shorter than 280  $m\mu$  was used during the radiation of the ergosterol. This exceptional destruction of vitamin was accompanied by the production of fractions showing unusually great absorption at 290  $m\mu$ .

#### *Volatility of Vitamin D.*

The distribution of antirachitic activity among the various fractions of the distillate is surprisingly uniform, considering the errors of measurement. In

ble

Group.	Nature of original substance.	No.	Temperature of "boiler."	Duration of heating.	Percentage of whole which was distilled.	Antirachitic activity (usually based on three or four pairs of rats).				
						Of original substance.	Of most active fraction.	Of residue in boiler.	Mean activity of products and residue.	Percentage loss of activity of whole.
1	Ergosterol free resins allowed some contact with air after radiation.	D15	145	h. m.	32	130	170	38	56	50
		D44	142	3 0	54	200	240	91	120	40
		D50	145	3 0	49	180	300	33	89	44
		D25	145	1 20	32	100	280	12.5	41	59
2	Products of previous distillations.	D51	141	2 0	52	200	160	25	73	63
		D37	147	1 25	70	36	70	16	41	Gain 14 per cent. (presumably fictitious)
		D16	145	2 0	61	83	130	5	70	13
3	Ergosterol free resins carefully protected from air before distillation.	D48	143	3 0	27	100	No activity detected	activity detected	25	Very large
		D49	100	2 25	19.5	100	25	None detected	25	Over 75
		D52	144	2 25	35	200	83	9.1	26	87

every case the least volatile fraction, condensed at 120° to 140° C. is of relatively low activity, compared with the more volatile fraction, condensed at 100° to 110° C. In eight cases out of ten the activity rises to a maximum in the penultimate fraction (100° to 110° C.) and then falls sharply for the most volatile fraction (20° to 95° C.). These effects may be partly due to the destruction of vitamin, after distillation, being greater in the hotter portions of the tube. We think, however, that this factor is not the dominant one, and that the vitamin is one of the more volatile constituents, but not the most volatile.

A distillation of a mixture of ergosterol and its radiation products showed no great difference between the volatility of the two, the highest concentration of ergosterol appearing in the fraction which usually shows highest vitamin content.

#### *Effect of Distillation on Absorption.*

Typical effects can be seen in figs. 3 and 4. In fig. 3 the dotted curve shows the absorption of an ergosterol-free resin which had been carefully screened from oxidation before distillation. The other curves correspond to the various fractions and the residue after distillation. Fig. 4 shows a similar set of data for an ergosterol-free resin which had been freely exposed to air before distillation.

It is clear that the processes of distillation and fractional condensation result in a considerable separation of absorbing from non-absorbing substances in the mixtures concerned. The ratio of absorption coefficients at 290  $m\mu$  for fraction 1 (130° to 140° C.) to fraction 4 (20° to 95° C.) is frequently more than 3 to 1, and rarely less than 2 to 1, and this probably gives too low an estimate of the degree of separation of substances. There is thus a clear tendency for a substance showing intense absorption at 290  $m\mu$  to collect in the least volatile fraction of the distillate. It is difficult to say how far the absorption of the products of distillation is due to the separation of products originally present in the mixture, and how far to the formation of new substances by heat, but we think the following conclusions are not improbable:—

1. That the most volatile fraction (of low absorption) represents substances originally present in the mixture. The evidence for this is that the redistillation of the residue from a previous distillation gives very little of a fresh volatile product, while the redistillation of a fraction previously condensed at 100° to 110° C. gives an appreciable fraction condensing below 95° C. This shows that if the volatile fraction is a

decomposition product at all, it is only formed by the early destruction of one constituent of the mixture.

2. That the substance showing intense absorption at 290  $m\mu$  is formed chiefly during distillation, and may or may not be originally present to some

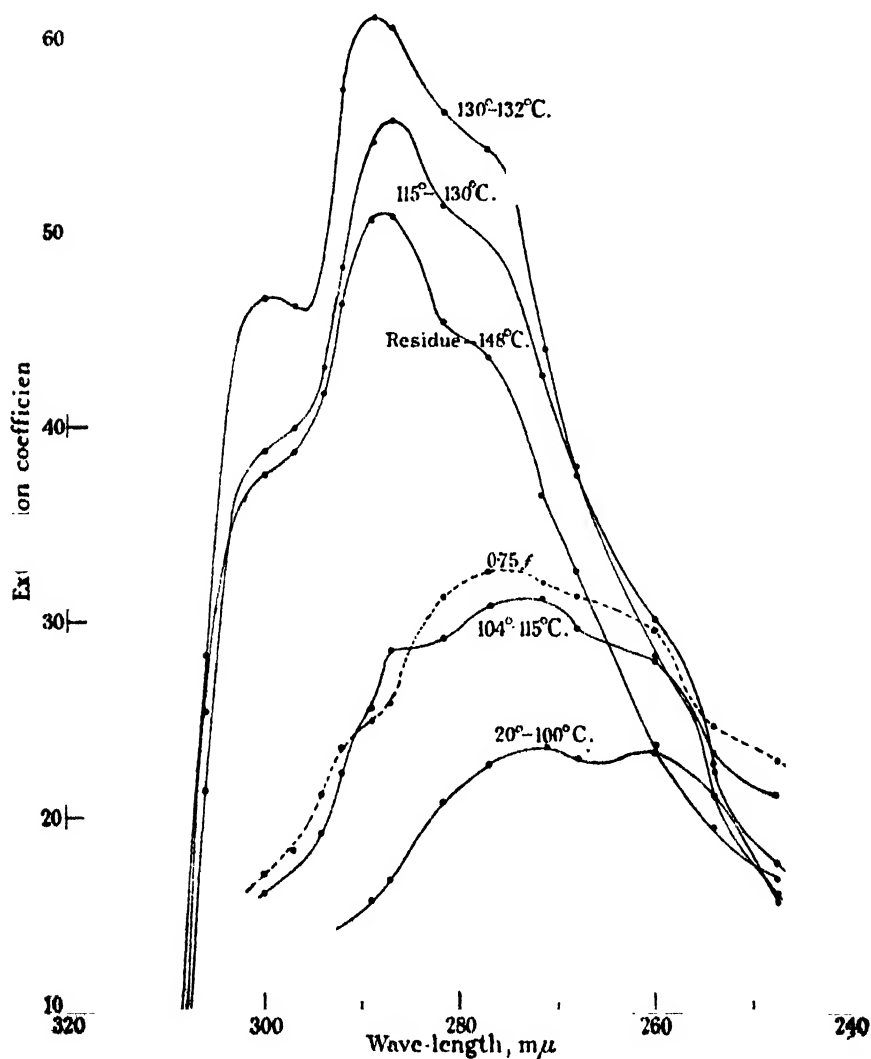


FIG. 2.—Absorption of the distillation products of an ergosterol-free resin protected from air. The dotted curve shows the absorption of the product 075f before distillation. The figures on the other curves show the temperature at which the various products were condensed.

extent in the mixture. This substance is apparently identical with that described by Windaus (1930) as formed by the thermal decomposition of the irradiation products of ergosterol at 200° C.

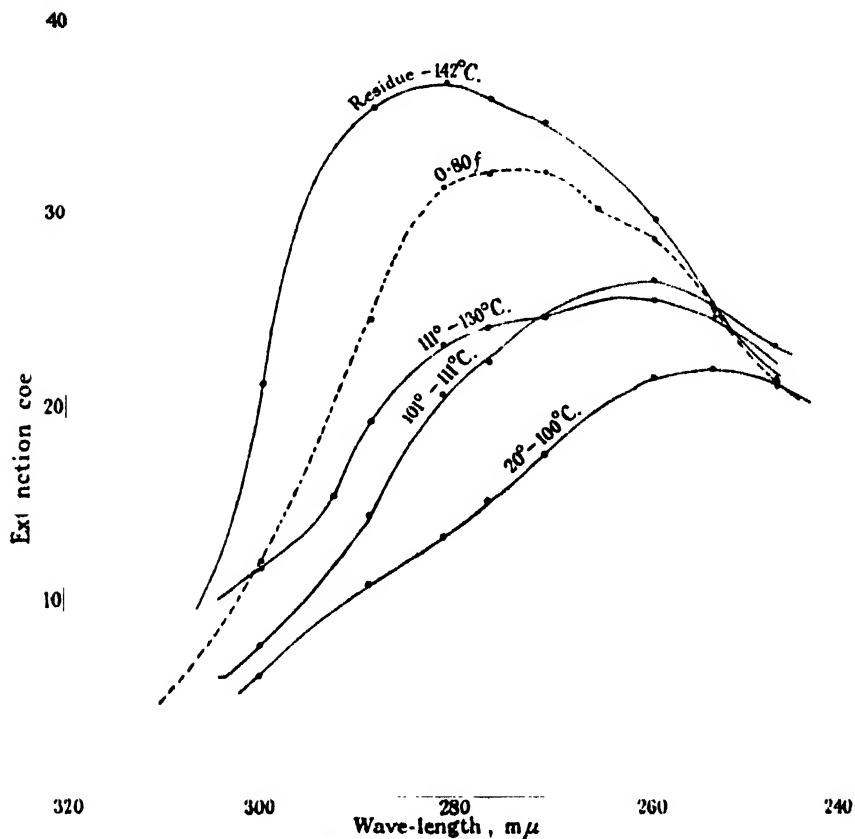


FIG. 4.—Absorption of the distillation products of an ergosterol-free resin not protected from air. The dotted curve shows the absorption of the product 080f before distillation. The figures on the other curves show the temperatures at which the various products were condensed.

Fig. 5 shows the absorption of the mixture containing the highest concentration that we have yet observed of the substance absorbing at 290 mμ. The intense absorption is very striking when compared with the curves for oxycholesterilene, and for ergosterol, which are drawn to the same scale.

We have no definite proof of the conditions governing the formation of this substance, but can say that, in our experiments, it has been formed in reduced amount, and in greatly reduced concentration, in the distillation of products which had been exposed to air before distillation. In products radiated

through filters (excluding wave-lengths shorter than  $280\text{ m}\mu$ ) and carefully screened from air, this absorbing substance has been formed in large amount and in high concentration.

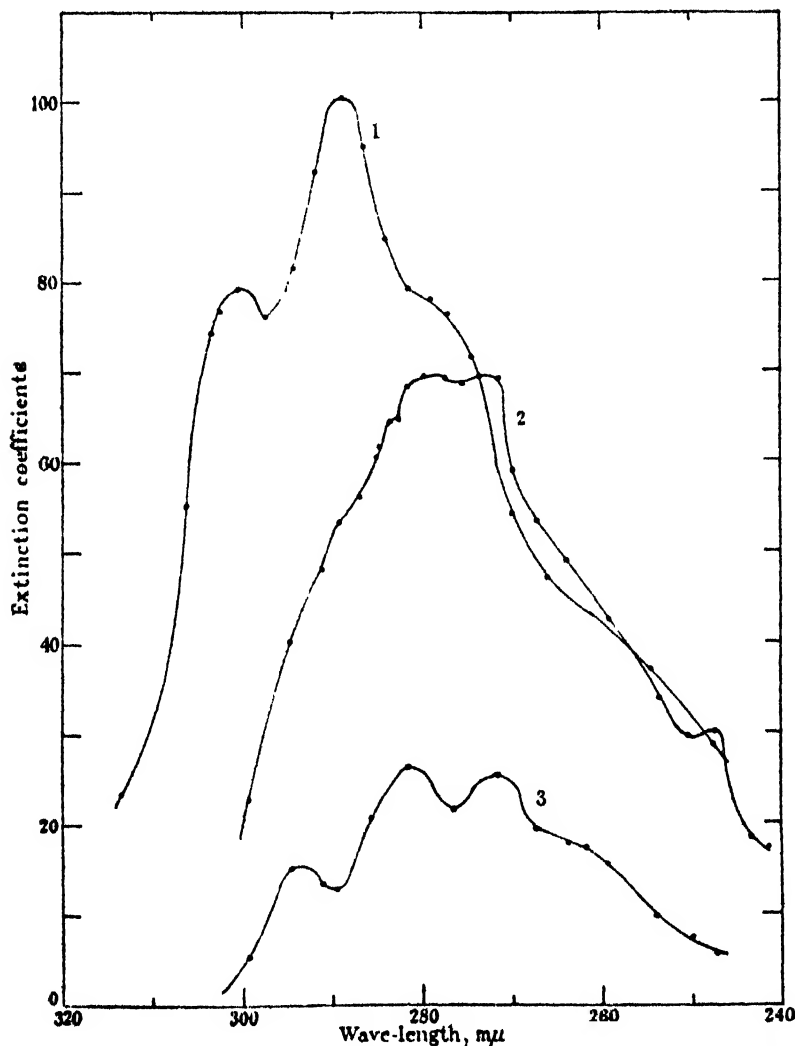


FIG. 5.—Absorption of product showing very high extinction coefficient at  $290\text{ m}\mu$ , contrasted with oxycholesterilene and ergosterol. Curve 1—Distillate. Curve 2—Oxycholesterilene. Curve 3—Ergosterol.

The total gain or loss of absorption on distillation has been measured in 20 experiments by adding together the products of the absorption and the weight of each fraction (including the residue), and comparing the sum with



the absorption before distillation. The largest gain is 60 per cent. and the largest loss is 57 per cent., while the majority of experiments show much smaller changes. In all the six cases observed of redistillation of one fraction of a previous distillate, there has been a definite loss of total absorption.

#### *Efficiency of the Distillation Process.*

It may be concluded that the process of distillation and fractional condensation described above is of some use for effecting a partial separation of the vitamin from other irradiation products of ergosterol, but is only of limited use in its present form. It is more effective as a means of concentrating the substance showing intense absorption at 290  $m\mu$ .

Further tests of the method have shown that it effects a partial separation of products having different solubilities in aqueous alcohol. The more volatile fractions are soluble in alcohol containing a higher percentage of water.

Tests made on the distillation of mixtures of equal weights of ergosterol and cholesterol, under the conditions described above, show a partial fractionation on condensation, the most volatile fraction containing 16 per cent. to 30 per cent. of ergosterol, and the least volatile 56 per cent. to 66 per cent. There was only a very small destruction of total absorption (1 per cent. to 3 per cent.), suggesting that decomposition was not serious. In these tests the separation would be expected to be of reduced efficiency, since the process was sublimation instead of distillation, and hence fractionation during the transformation to vapour could not occur. In the distillation of the resins no solid phase is involved, and we believe that partial separation occurs during the volatilisation of the liquid.

#### *Nature of Changes occurring during Distillation.*

Some of the facts above described are difficult to explain by any, as yet, accepted theory. The point of special difficulty, as well as interest, is that concerned with the formation, during heating *in vacuo*, of the new substance with intense absorption at 290  $m\mu$ . This substance has no antirachitic activity in itself, but its appearance is in some way associated with the disappearance of vitamin. It cannot, however, be regarded as a simple product of vitamin destruction, since its production bears no direct relation to the vitamin content shown, before distillation, by the irradiation products from which it is formed. The two conditions which appear to favour its formation, and the concurrent disappearance of the vitamin, are previous protection of the irradiation product

from exposure to air, and the exclusion of short wave-lengths during the original irradiation. These facts could be explained by the following hypothesis which we put forward very tentatively. We suggest that one of the initial radiation products of ergosterol is an unstable substance as described by Holtz (1929) and Windaus (1930) (Kurzbestrahlungsprodukt), and that this has low absorption and no antirachitic activity; that this substance is very readily destroyed by absorption of oxygen, or by radiation of short wave-lengths; but that, if not previously destroyed, it is converted by heat into the substance absorbing at 290  $m\mu$ , with an associated destruction of part of the vitamin present. As to the meaning of this association, our evidence does not entitle us to offer even a suggestion. We can only record it as having occurred with apparent regularity in our experience hitherto.

#### *Summary.*

1. A method is described for the partial separation of the irradiation products of ergosterol, by distillation and fractional condensation in a high vacuum.

2. By double distillation, followed by crystallisation from aqueous alcohol, a crystalline product has been obtained, showing high antirachitic power, and a melting point of 113° to 115°.

3. It is not suggested that these crystals are pure vitamin D. They may consist of an inactive substance contaminated with an extremely active vitamin, or of crystalline vitamin forming mixed crystals with an inactive substance. It is possible, on the other hand, that several antirachitic substances are formed by the irradiation of ergosterol, and that the crystals are a relatively pure specimen of one of these substances.

4. Some evidence is given as to the degree of efficiency of the process of fractional condensation in a high vacuum.

We wish to express our thanks to Dr. O. Rosenheim and Dr. R. K. Callow for suggestions in connection with this work, and for the preparation of highly purified ergosterol by recrystallisation of the benzoate.

The ergosterol used in this work was kindly supplied on special terms by Messrs. Boots, Messrs. British Drug Houses, and Messrs. Burroughs Wellcome & Co.

#### REFERENCES.

- Bourdillon, R. B., Fischmann, C., Jenkins, R. G. C., and Webster, T. A. (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 561.  
Bourdillon, R. B., Gaddum, J. H., and Jenkins, R. G. C. (1930). 'Proc. Roy. Soc.,' B, vol. 105, p. 388.

- Holtz, F. (1929). 'Strahlentherapie,' vol. 34, p. 637.  
Reerink, E. H., and Van Wijk, A. (1929). 'Biochem. J.,' vol. 23, p. 1294.  
Webster, T. A., and Bourdillon, R. B. (1928). 'Biochem. J.,' vol. 22, p. 1223.  
Windaus, A., and Holtz (1927). 'Nachr. Ges. Wiss. Gött., Math. Phys. Kl.,' p. 2.  
Windaus, A. (1930). 'Nachr. Ges. Wiss. Gött., Math. Phys. Kl.,' p. 36.

*Note on the Difficulty of Comparing Results from Different Laboratories.*

There is at present no accurate basis for the comparison of the antirachitic activities of the purest specimens of vitamin D obtained by workers in different research laboratories. Although many authors have stated the doses of their preparations which would cure rickets in rats fed on some definite diet, such statements afford only a very rough basis of comparison, owing to variations in the standard of "cure" adopted, and to the wide variations in the sensitivity of rats fed on diets which are apparently similar. In our own series of tests we find a large seasonal variation in the sensitivity of rachitic rats to a standard solution of vitamin D, even when both the test rats and their parents are on carefully standardised diets. This variation is of the order of 5 : 1, even on a comparison by monthly averages of litters. This, and other sources of variation, may together produce a difference of fully 10 : 1 between the activities of preparations, which, from the data given by workers in different laboratories, would appear to have equal curative power. In our own work we attempt to counteract the effects of seasonal variations by comparing each unknown solution with a standard solution in olive oil kept at 0° C., and to meet the variations from litter to litter by comparing the standard and unknown solutions on litter mates in each test. We have reasons (which will be published shortly) for believing that such a standard solution will remain almost unchanged in vitamin content, for years at 0° C., and for long periods at laboratory temperatures.

Probably other workers have adopted a similar procedure for avoiding variation in their own tests; but in order to make the results of different laboratories comparable, it is clearly desirable that one such arbitrary, but stable, standard should be made available to all concerned in this field of research.

## *The Effects of Further Irradiation of the Radiation Products of Ergosterol.*

By F. A. ASKEW, R. B. BOURDILLON, H. M. BRUCE, R. G. C. JENKINS, and  
T. A. WEBSTER, National Institute for Medical Research.

(Communicated by H. H. Dale, Sec. R.S.—Received August 5, 1930.)

The effects of ultra-violet radiation upon ergosterol have been studied by many workers, but the results have been difficult to interpret owing to the fact that certain of the products of radiation are themselves changed by further exposure, at a rate greater than the velocity of change of ergosterol itself.

Windaus, Westphal, Werder and Rygh (1929) have carried out some experiments in which the irradiation products of ergosterol were freed from unchanged ergosterol, and then re-radiated. They used the same source of energy (a magnesium spark) for both first and second irradiations, and obtained results of some interest, but still difficult to interpret.

We have carried out a series of experiments on re-radiation, by exposing ergosterol to radiation of long wave-lengths only, removing the unchanged ergosterol, and then exposing the product to radiation of either short or long wave-lengths. These tests have been supplemented by the further irradiation of such isolated fractions of the mixed irradiation products of ergosterol as we have been able to separate from the mixture by precipitation or distillation. The absorption spectra and antirachitic activity of the products were measured both before and after the second irradiation.\*

### *Technique of Measurement and of Irradiation.*

The measurements of absorption spectra, and of antirachitic activity were made by the methods described in our paper on the Distillation of Vitamin D (Askew *et al.*, 1930).

The initial irradiations of ergosterol were carried out on alcoholic or ethereal solutions of highly purified ergosterol. These solutions were irradiated by a mercury vapour lamp, through light filters of different substances, as shown in the second column of Table I. During irradiation the solution was either contained in silica cells in a layer 1 cm. thick and stirred by nitrogen, or was passed in a rapid stream through a spiral silica tube of 2 mm. bore, which

\* A preliminary account of certain of these experiments was published in 'Nature' (1930).

surrounded the lamp, and was cooled by water. From 20 to 40 per cent. of the ergosterol was destroyed during this irradiation. The unchanged ergosterol was then removed by digitonin as described in a previous paper (Webster and Bourdillon, 1928). The second irradiations were always carried out on alcoholic solutions of the substances concerned, in small silica cells which held a layer 1 cm. thick between flat polished walls. The cells were placed 15 cm. from a K.B.B. mercury vapour lamp taking 2.5 amps. and 120 volts at atmospheric pressure. In most experiments one of the following light filters was placed between the lamp and the solution.

- A. A silica cell containing a 1 per cent. solution of xylene in alcohol. This filter was 1 cm. thick and transmitted about 90 per cent. of the radiation of wave-lengths longer than  $280\text{ m}\mu$ , but cut off radiation of wave-lengths shorter than  $275\text{ m}\mu$  almost completely. As the absorbing power of this filter increased on prolonged exposure, the solution was renewed frequently. The radiation transmitted by this filter is referred to in this paper as "long waves."
- B. Two separate silica cells each with polished flat walls 2.5 cm. apart. One contained chlorine gas, and the other bromine vapour. The combined effect of these filters was to transmit about 30 per cent. of the radiation of wave-lengths shorter than  $270\text{ m}\mu$ , but to cut off almost all radiation of wave-lengths between 300 and  $600\text{ m}\mu$ . Radiation of wave-lengths between  $280\text{ m}\mu$  and  $300\text{ m}\mu$  was transmitted in greatly reduced intensity. The radiation transmitted by this filter was thus chiefly of wave-lengths between  $210\text{ m}\mu$  and  $280\text{ m}\mu$ , and is referred to in this paper as "short waves."

*Types of Product subjected to Re-radiation.*

Three types of product have been used for these experiments. Firstly, the ergosterol-free resins obtained by the methods described above; secondly, a granular solid substance prepared from these resins by partial precipitation from alcoholic solution by the addition of water; and thirdly, the crystals described in our preceding paper on "Distillation." These three classes of experiment will be considered separately.

*1. The Re-radiation of Ergosterol-free Resinous Mixtures.*

The results of these experiments are shown in Table I, and the absorption changes observed in one series of tests are shown in fig. 1. Curve A shows

Table of Antirachitic Activity

First radiation.				Second radiation.						
Light filter (1 cm. thick).	Absorption.		Antirachitic activity.	Light filter.	Concen- tration of solution.	Time.	Absorption.		Antirachitic activity.	Percentage fall in activity.
	At 281 m $\mu$ .	At 271 m $\mu$ .					At 281 m $\mu$ .	At 271 m $\mu$ .		
1 per cent. xylene in alcohol.	22.2	23.4	160	Chlorine and bromine ... Xylene 1 per cent. .... None .....	per cent. 0.01 0.01 0.01	mins. 15 15 5	39.25 19.25 31.0	35.9 20.8 28.0	55 160 140	65 0 10
1 per cent. xylene in alcohol.	16.8	18.2	140	Chlorine and bromine ... Xylene 1 per cent. .... None .....	0.01 0.01 0.01	15 60 10	24.0 10.0 16.1	22.0 11.8 16.3	45 140 33	68 0 76
50 percent. NiSO <sub>4</sub> . 7H <sub>2</sub> O in water, 1 per cent. xylene in alcohol.	16.8	19.0	110	Chlorine and bromine Xylene 1 per cent. ....	0.01 0.01	15 15	35.6 14.7	31.1 16.4	33 100	63 9
50 percent. NiSO <sub>4</sub> . 7H <sub>2</sub> O in water, 0.2 per cent. phenol.	14.7	18.4	150	Chlorine and bromine ... Xylene 1 per cent. ....	0.01 0.01	15 15	34.2 —	29.7 —	25 125	83 17
50 percent. NiSO <sub>4</sub> . 7H <sub>2</sub> O in water.	48.6	43.5	75	Xylene 1 per cent. ....	0.0025	85	9.1	10.2	42	44
Chlorine gas (2.5 cm.) — Bromine vapour (2.5 cm.)	27.4	26.6	100	Chlorine and bromine...	0.01	15	34.4	29.1	42	58

the absorption of the product obtained by irradiation of ergosterol with "long waves," which destroyed 30 per cent. of it, and subsequent removal of the unchanged ergosterol. The antirachitic activity of this product was 160, using the notation described in our paper on "Distillation" (*loc. cit.*). Curve C shows the absorption of the product after a second radiation for 15 minutes by "short waves." The antirachitic activity was 55, and had thus fallen to about one-third of its former value, although the absorption at 280  $m\mu$  had

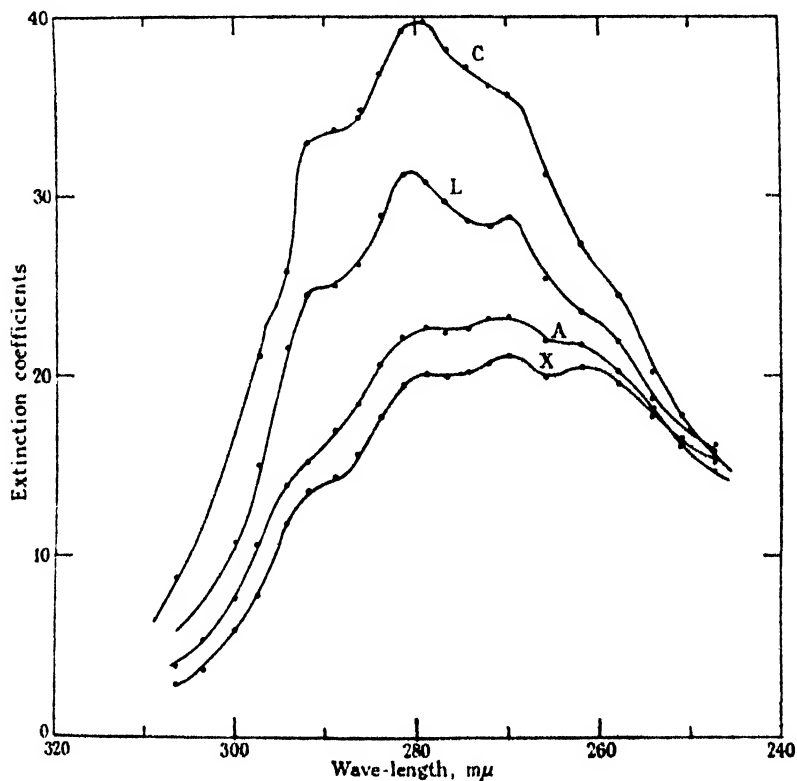


FIG. 1.—Changes in absorption produced by the irradiation of an ergosterol-free resin (curve A) initially irradiated by long waves

Curve.	Type of second radiation.	Antirachitic activity of product.
A	None .....	160
C	Short waves .....	55
X	Long waves .....	160
L	Unfiltered mercury arc.....	140

nearly doubled. Curve X shows the result of re-radiation of another sample of the solution for 15 minutes by long waves, as used for the first radiation. This caused a small fall in absorption, without perceptible change in anti-

rachitic activity. Curve L shows the effect of re-radiation of a third sample for 5 minutes without any light filter. In this case there was a rise in absorption with a very slight apparent fall in antirachitic activity, to 140 from the original 160.

This experiment has been repeated several times with the results shown in Table I (p. 93).

The antirachitic data in this table depend on tests, in which the degrees of healing, on which an estimate of activity could be based, were obtained in only two to four pairs of rats for each substance tested. They are thus liable to appreciable error, as is suggested by the variable effects on antirachitic activity caused by the re-radiations through xylene filters. It is, however, quite clear that re-radiation with short waves causes a rapid loss of antirachitic activity accompanied by a large rise in absorption at  $280\text{ m}\mu$ . On the other hand, the fall in activity on re-radiation with wave-lengths longer than  $280\text{ m}\mu$  is much smaller, as would be expected from the results obtained by other workers on the direct irradiation of ergosterol.

#### *Inferences as to the Absorption Spectrum of Vitamin D.*

In the solutions re-radiated by "short waves" there is a large rise in absorption at  $280\text{ m}\mu$  with a simultaneous fall in antirachitic activity. This seems to give definite proof that the substance absorbing at  $280\text{ m}\mu$  is not vitamin D. The general similarity of curve C in fig. 1 to curves previously attributed by Bourdillon *et al.* (1929) to a "substance A," suggests strongly that we are dealing with the same substance. These results afford definite confirmation of many of the conclusions reached by Reerink and Van Wijk (1929), in a very illuminating paper which appeared just after we had begun these experiments. Hence, we agree with the suggestion of these authors that the substance showing maximal absorption at  $280\text{ m}\mu$  (substance A) is produced by radiation of short wave-lengths, and is not vitamin D. Our "substance A" is probably identical with their "substance S." In the solutions re-radiated by long waves there is a slow fall in absorption at  $280\text{ m}\mu$  and at  $270\text{ m}\mu$ , usually accompanied by a slow fall in antirachitic activity.

In the present state of knowledge we would hesitate to attribute any definite absorption curve to vitamin D, although our later work confirms that of Reerink and Van Wijk (1929), and of Windaus (1930), in so far as it shows some correlation between absorption at  $270\text{ m}\mu$  and antirachitic activity. This correlation is very conspicuous in the case of the crystals described in



our paper on "Distillation" (*loc. cit.*). However, the ease with which the absorption at 270  $m\mu$  can be apparently increased, by the presence of smaller quantities of substances with high absorption maxima at 280  $m\mu$  or 290  $m\mu$  makes the study of absorption of doubtful utility for the estimation of vitamin D in mixtures.

## 2. *The Granular Solid obtained by Precipitation from Aqueous Alcohol.*

*Method of Production.*—Ergosterol free resins are prepared by a single radiation as described above, and then dissolved in alcohol to a concentration of about 1 per cent. Water is added till the liquid is slightly turbid and the mixture is left in a vacuum desiccator with sulphuric acid, or ethyl hydrogen sulphate, so that it evaporates slowly. This usually causes the deposition of a granular solid, which is followed by a liquid oil if evaporation proceeds too far. The solid can be separated by decantation, and partially purified by solution in alcohol and reprecipitation with water. The yield that we have obtained has been irregular, and it is not impossible that oxygen plays a part in the formation of this product. Our best yield was obtained after radiation of ergosterol through a filter of nickel sulphate.

*Properties.*—This product forms a nearly colourless, flocculent precipitate, rapidly settling after shaking. Under low magnifications it appears crystalline, but, if highly magnified, it is seen to consist of agglomerates of particles with rounded corners and irregular shape.

When separated from the mother liquor by decantation, this product only dissolves slowly in absolute alcohol, in marked contrast to the original resin.

*Antirachitic Activity.*—The apparent antirachitic activity of the six specimens that we have examined varied from a maximum of 33 to a minimum of 12, which was given by three specimens, including that of which the absorption is shown in fig. 2. As there is no reason to think that these granular deposits are a single pure substance, we have little hesitation in ascribing the observed antirachitic activity to vitamin carried down with the inactive material, of which the precipitate presumably consists, rather than to any inherent property of the precipitate itself.

*Absorption Spectrum.*—The upper curve in fig. 2 shows the absorption spectrum of a sample of this product that had been twice re-dissolved and re-precipitated, and then washed with 50 per cent. alcohol. Of the six samples that we have prepared on different occasions, all gave absorption curves with a similar maximum at 251  $m\mu$ , but four of the six showed a rather flatter curve

with a lower maximum, apparently owing to greater contamination with a non-absorbing substance.

**Effect of Re-radiation.**—The granular substance shows a surprising sensitivity to ultra-violet radiation, whether of long or of short wave-lengths. On re-radiation in 0.0025 per cent. alcoholic solution by short wave-lengths the absorption measured at 255  $m\mu$  fell to one-quarter of its value in 90 seconds; and even when long waves were used for the re-radiation, the absorption fell to one-half of its value in less than 4 minutes. These results are shown by the series of curves in fig. 2.

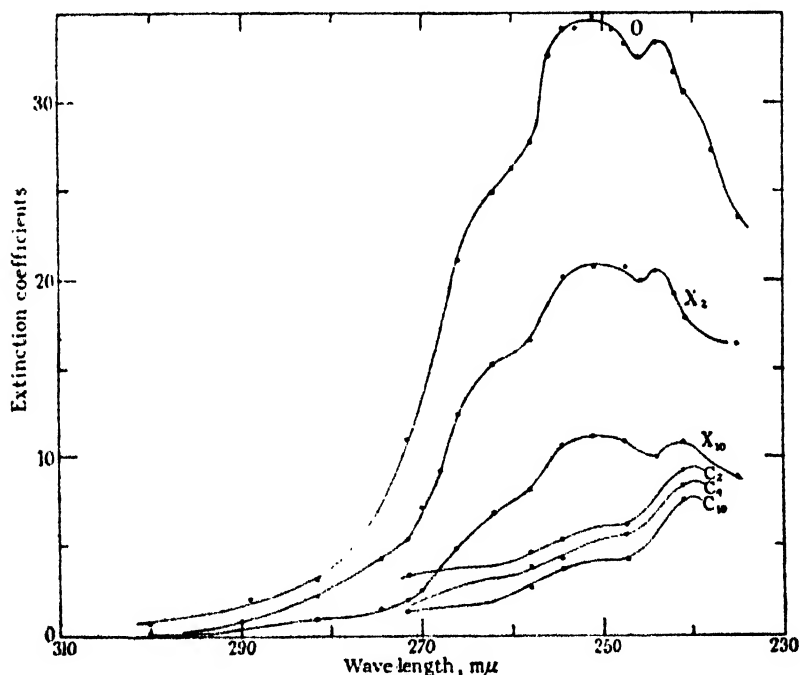


FIG. 2.—Absorption of granular solid product, and changes on re-radiation.

O	...	Before second radiation.
X2	...	After 2 minutes' radiation by long waves.
X10	...	After 10 minutes' radiation by long waves.
C2	...	After 2 minutes' radiation by short waves.
C4	...	After 4 minutes' radiation by short waves.
C10	...	After 10 minutes' radiation by short waves.

### 3. Re-radiation of Crystalline Product obtained by Distillation.

The crystalline products obtained by distillation (*loc. cit.*) were also re-radiated, and appeared to be far less sensitive to short waves than the granular solid,

although there are indications that the apparently slow rate may be due to the formation of a relatively stable new product.

Fig. 3 shows the rates of fall in absorption of the crystals, of the granular solid and of ergosterol itself, when irradiated under corresponding conditions by short waves.

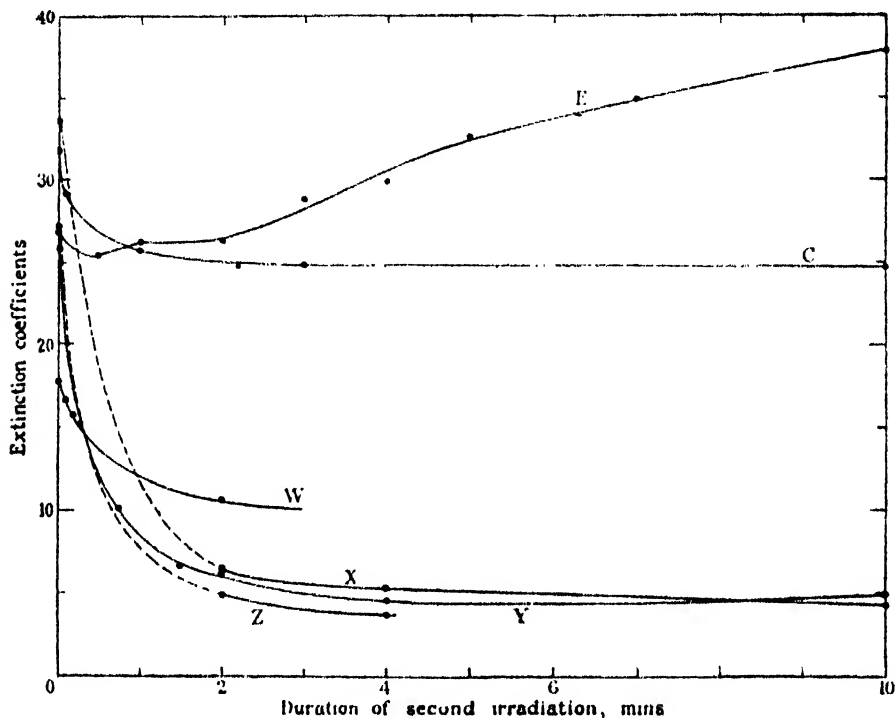


FIG. 3.—Changes in absorption of various products during irradiation by short waves in 0.0025 per cent. solution. E, ergosterol; this curve reaches its highest point after 15 minutes' irradiation and then falls slowly. C, crystals obtained after distillation. W, X, Y, Z, separate specimens of granular solid.

The dotted lines represent the hypothetical values for two curves where no measurements were made during the first 2 minutes of irradiation. For curves E and C the absorption measurements were made for the wave-length  $281\text{ m}\mu$ . For the other curves measurements were made for wave-lengths close to the maximum of absorption at  $251\text{ m}\mu$ .

There is a characteristic difference between the slopes of the curves for these three substances, which shows that this procedure forms a useful means of distinguishing some of the irradiation products of ergosterol, even when in an impure state. It has the advantage over the simple measurement of an absorption spectrum, of being less easily confused by the presence of small quantities of impurities.

*Evidence for Direct Conversion of Ergosterol to an Unstable Substance formed simultaneously with Vitamin D.*

In the course of this work we have made observations which support the hypothesis of the direct formation from ergosterol of an unstable product of no antirachitic activity. [Compare Holtz (1929), Windaus (1930), Askew, *et al.* (1930)]. Thus...

1. When preparations initially irradiated by long waves have been re-radiated by short waves, the rise in absorption at 280  $m\mu$  has varied greatly in amount, but has shown no correlation with the antirachitic activity of the initial product. This seems to show that the "substance A" (or "S") formed in the second radiation is produced not from vitamin D, but from another substance, present in the solution in quantity varying according to the exact conditions of preparation of the product.

2. We have never observed any rise in antirachitic activity on re-radiation of a solution from which ergosterol had been removed. If vitamin D were formed from ergosterol in two stages, a rise in activity would have been expected in some at least of our experiments. This suggests that the hypothetical unstable substance is not a precursor of the vitamin, but is formed simultaneously with it.

3. In our own experience, and in that of Windaus (1930), the radiation products of ergosterol absorb oxygen on exposure to air very rapidly, whereas they only slowly lose antirachitic activity. This suggests the presence of some easily oxidised product other than vitamin D.

4. If a series of preparations are made by irradiating ergosterol for different periods, and then removing the unchanged ergosterol, the antirachitic activity per unit weight of the products increases as the period of irradiation is decreased, until the latter is only long enough to destroy a moderate fraction of the ergosterol (say 30 per cent. if the solution is weak and well stirred). Further decrease in the period of irradiation does not in our experience produce more active products. It only gives a smaller yield of a product of the same activity. These results may be due to experimental errors, or to a special instability of the vitamin when nearly pure. We think it more probable, however, that the true explanation is the simultaneous formation from ergosterol of vitamin D and of an inactive product, in a constant ratio to one another. This would prevent the concentration of vitamin from ever rising above the limit determined by that ratio, even if the period of irradiation was so short that practically no vitamin was destroyed.

*Summary.*

1. The effects of a second irradiation by short waves on the substances formed by a first irradiation of ergosterol by long waves have been studied. The results show that the product with high absorption at  $280\text{ m}\mu$  is not vitamin D as previously suggested.

2. The substance with high absorption at  $280\text{ m}\mu$  can be formed by the action of short-wave radiation on some product of a previous action of long waves on ergosterol.

3. Reasons are given for supposing that the initial effect of long-wave radiation on ergosterol is the simultaneous production of at least two substances, only one of which is vitamin D.

We wish to express our thanks to Dr. O. Rosenheim and Dr. R. K. Callow for suggestions in connection with this work, and for the preparation of highly purified ergosterol by recrystallisation of the benzoate.

REFERENCES.

- Askew, F. A., Bourdillon, R. B., Bruce, H. M., Jenkins, R. G. C., and Webster, T. A. (1930) 'Proc. Roy. Soc.,' B, vol. 107, p. 76.
- Bourdillon, R. B., Fischmann, C., Jenkins, R. G. C., and Webster, T. A. (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 561.
- Bourdillon, R. B., Jenkins, R. G. C., and Webster, T. A. (1930). 'Nature,' vol. 125, p. 635.
- Holtz, F. (1929). 'Strahlentherapie,' vol. 34, p. 637.
- Reerink and Van Wijk (1929). 'Biochem. J.,' vol. 23, p. 1204.
- Webster, T. A., and Bourdillon, R. B. (1928). 'Biochem. J.,' vol. 22, p. 1223.
- Windaus, A., Westphal, K., Werder, F. v., and Rygh, O. (1929). 'Nachr. Ges. Wiss. Gott., Math. Phys. Kl., p. 46.
- Windaus, A. (1930). 'Nachr. Ges. Wiss. Gott., Math. Phys. Kl., p. 36.

*The Vital Staining of Normal and Malignant Cells.*

III. *Vital Staining of Acinar Cells of the Pancreas and its Bearing on the Theories of Vital Staining with Basic Dyes.*

By R. J. LUDFORD.

(From the Laboratories of the Imperial Cancer Research Fund.)

(Communicated by J. A. Murray, F.R.S.—Received June 24, 1930.)

[PLATE 9.]

1. *Introduction.*

Is there a fundamental difference between vital staining with acid, and with basic dyes? Upon the answer to this question depend some of the most controversial problems which are occupying the minds of cytologists at the present time. I have endeavoured to contribute towards the solution of this problem by a study of the acinar cells of the pancreas of the mouse. The pancreas cell offers considerable advantages for such a study, owing to the ease with which its cytoplasmic structure can be demonstrated, and its ready stainability with basic dyes. Furthermore, this cell has received much attention from those interested in the cytology of secretion and its relation to vital staining.

2. *Theories of Vital Staining with Basic Dyes.*

In previous communications (Ludford, 1928, 1929) I have discussed the present position of our knowledge of the cellular mechanism involved in vital staining with acid dyes, such as trypan blue and carmine. According to von Möllendorff (1920) vital staining with basic dyes differs essentially from vital staining with acid dyes. He believes that while acid dyes are segregated in cells and so give rise to new formations—vacuoles and granules—basic dyes differ in that they stain preformed structures. This conception is based upon the fact that it is possible to stain supravitaly with certain basic dyes phagocytosed cell inclusions, the yolk granules of some embryonic tissues, and acid dye droplets which have been formed in cells subsequent to the injection of acid dyes. von Möllendorff (1920) carried out a large number of experiments with basic dyes to determine what properties favoured vital staining. He found that the best basic dyes for staining cell granules were those which were

only slightly lipoid soluble, but were readily flocculated in contact with acid colloids. To this class belong neutral red, cresyl blue, methylene blue and Bismarck brown. Basic dyes which are readily soluble in lipoids, and show little or no tendency to be flocculated by acid colloids, produce only a diffuse staining of cells. Rhodamin and Diamant-fuchsin are examples of this type. Some striking exceptions occur amongst this latter group, of which the specific staining of mitochondria with Janus green is well known. Various attempts have been made to account for this. It has been suggested that the mitochondria are altered when they stain, and Fauré-Frémiet has attributed their staining to the solubility of the Janus green in their lipoid constituent. Cowdry (1918) has, however, pointed out that the efficacy of the Janus green as a mitochondrial stain depends upon its specific chemical constitution, so that it seems probable that a chemical reaction may be involved in this process.

It was upon von Möllendorff's conception of the mode of action of basic dyes such as neutral red that Parat based his theory of cell structure. According to Parat's (1925) earlier papers, the cytoplasm contains two universally present organellæ—the mitochondria and the vacuome. Mitochondria can often be seen clearly in the living cell. They stain supravitaly with Janus green B, and Janus black (Hoechst). They can be demonstrated in fixed material by methods which are known to demonstrate such lipins as lecithin. Giroud (1929) has adduced microchemical evidence to show that mitochondria consist of a lipoid and a protein constituent.

The "vacuome" is seen as a series of droplets when cells are vitally stained with the granule-staining basic dyes, preferably with neutral red. Their general arrangement in the cell coincides with the form of the Golgi apparatus as demonstrated by the osmic or silver impregnation methods. When, for example, nerve cells are stained vitally with neutral red, the vacuome is seen as a series of red droplets which, if joined together would give the classical picture of the "internal reticular apparatus" of Golgi. Parat believes that the Golgi apparatus, as revealed by cytological methods, may arise as the result of a fusion of such linearly arranged droplets or granules, brought about by the treatment to which the tissues have been subjected. Covell and Scott (1928) who have studied the problem in the nerve cells of the mouse state that their findings offer strong support to Parat's theory. The observations of Cowdry and Scott (1928) on the vital staining of the protozoan, *Plasmodium præcox*, also appear confirmatory of Parat's views. Wen-Chao Ma and his collaborators (1928, 1929) have described the result of supravital staining with neutral red, as well as with a mixture of neutral red and Soudan III.

It is not clear from their papers whether they believe the whole Golgi apparatus, or only a part of it, is stained in this way. The view is expressed, however, that the staining is brought about by the dyes dissolving in the lipid constituent of the Golgi apparatus.

In his more recent papers Parat (1928) has modified his original theory. He now speaks of a "Zone of Golgi." This is comprised of the "vacuome" with which is associated certain specialised mitochondria, which he calls "chondriome actif" or dictyosomes. These are applied to the surface of the vacuoles, and around this area of the cytoplasm lipins accumulate as the result of their property of lowering surface tension. A dictyosome applied to the surface of a vacuole constitutes an oil-water interface system. Here molecules of amino-acids become orientated in a manner previously suggested by Brailford Robertson. The condensation of molecules thus brought about facilitates synthesis of proteins. The elaborated products collect in the vacuoles, condense into granules, and thus the secretion granules arise in gland cells. The "zone of Golgi" thus becomes a specialised area in the cytoplasm specially concerned with synthetic processes. The advent of the "chondriome actif" introduces a second source for the origin of the typical reticulate form of the Golgi apparatus. It is assumed that it may in some cases be due to an intense impregnation of the "chondriome actif" with osmic acid or silver. This idea has also been developed by Feyel (1928) in a study of the vitally stained and impregnated cells of the kidney of amphibians and fish.

Zweibaum and Elkner (1930), who have reinvestigated the problem of the Golgi apparatus in tissue cultures, accept Parat's views that it arises as the result of either an impregnation of the coalesced droplets which constitute the vacuome, or by an intense impregnation of certain of the mitochondria localised in the area of the sphere. They deny that there exists a reticulate Golgi apparatus in living fibroblasts, which breaks up, as I have described (1927), when cells grown in vitro spread out on the surface of the cover slip. Their failure to confirm my results is probably due to the fact that my observations were made on tissues grown in a saline medium in which the cells flatten out much more than in the case of the cells Zweibaum and Elkner figure. Most of their observations, moreover, were carried out with plasma cultures where the spreading of the cells is not so marked. These authors do, however, figure the typical reticulate Golgi apparatus in fibroblasts. Studies on the connective tissues upon which I am at present engaged confirm my opinion that such a reticulate, or canicular, structure exists in the living cell, contrary to the belief of Zweibaum and Elkner, who consider it an artifact.



The researches so far discussed lend support to the view of Parat that there exists in the cell a system of vacuoles, stainable with neutral red, which constitute a fundamental part of the cellular mechanism. Within the last few years, however, a great deal of evidence has been brought forward refuting this conception. The earlier literature bearing on the problem is discussed in the valuable review of von Möllendorff (1920). In the light of recent developments, however, the views of Teichmann (1891) and Heidenhain (1908) are so pertinent as to merit consideration here. Teichmann studied the vital staining of the gut epithelium of the tadpole with methylene blue. He thought that when this dye entered the cells pathological changes ensued. Part of the protoplasm became separated from the colloidal system of the cell. It was this dead part of the protoplasm which stained with methylene blue. Heidenhain considered it improbable that the droplets that appear in nearly all cells after vital staining with basic dyes could have been present in the unstained cells, and yet have been invisible in good cytological preparations. He came to the conclusion that the coloured droplets were for the most part artificial products, which arose by a kind of internal secretory process so that the dye was precipitated in the protoplasm. More recently Chlopin (1927) has expressed somewhat similar views as the result of an investigation of the vital staining of a wide range of cells from invertebrates and lower vertebrates. He found that neutral red was able to stain preformed granules and droplets in cells as well as give rise to new inclusions. With intense staining the newly-formed dye droplets contain almost without exception varying amounts of an albuminous substance which in fixed tissue is stainable with basic aniline dyes. This substance Chlopin calls the "Krinom." Its formation is not accompanied by any marked disturbance of the normal physiological function of the cell, or by any demonstrable pathological deformation of its morphology. Krjukowa (1929) who investigated the vital staining of the salivary gland cells of the larva of *Chironomus* agrees with Chlopin that neutral red on entering the cells gives rise to new formations. Within the cells it follows "la voie sécrétoire," that is, the dye droplets are formed in relationship with the Golgi bodies, which Krjukowa points out are visible in the living cell. These observations are particularly interesting because it was the study of the vital staining of these cells that first led Parat to formulate his "vacuome" theory. Alexenko (1929) has recently published a report of the vital staining of neurones of the fowl at various stages of embryonic development. He also expresses the opinion that the droplets, which appear in these cells after staining with neutral red, arise in association with the Golgi apparatus. Weiner (1930)

has come to a similar conclusion from a study of the vital staining of oöcytes of earthworms, in which cells, as Gatenby (1926) has pointed out, the Golgi bodies are visible during life. A great deal of work has been done on the vital staining of germ cells by Gatenby and Hirschler (1928*a*, 1928*b*). This work has been fully discussed by Gatenby (1929), who has emphasised the fundamental difference between Golgi bodies and the "vacuome"; the latter he regards as being "primitively associated with, and probably produced by" the former. Gatenby and Wigoder (1929) have also shown that after x-radiation the Golgi bodies undergo changes, but the "vacuome" or vacuolar system is not affected.

This survey of the literature can be summarised by saying that the present position of the problem of the nature of vital staining with basic dyes is as follows :-

1. After vitally staining a wide variety of cells with neutral red, droplets appear in the cytoplasm arranged often in a definite manner which coincides with the position and form of the Golgi apparatus as demonstrated by the osmic and silver impregnation techniques.

2. It has been shown that neutral red in addition to staining preformed cell inclusions is capable of giving rise to new formations.

The question arises what structure, if any, exists in the living cell in that area of the cytoplasm where dye droplets appear after vital staining with neutral red? Upon the answer to this question will depend our ideas as to whether there is any fundamental difference in the reaction of the living cell towards acid and basic dyes. The experimental work, of which the description follows, was carried out in order to throw further light on these problems.

### *3. The Morphology of the Process of Secretion in the Acinar Cells.*

Numerous researches carried out within recent years by Nassonov, Bowen, Cramer, Ludford and others have established the fact that the secretion granules of gland cells arise in relationship with the Golgi apparatus. In the acinar cells of the pancreas of the mouse, as in most other gland cells which have been studied, the Golgi apparatus enlarges at the onset of secretory activity, spreads out in the cytoplasm and secretion droplets form in connection with it. The various stages of this process are seen in figs. 1 to 4 (Plate 9). Here is represented the origin of secretion granules seen in preparations made by my modification of the Kopsch technique (1926). Fig. 2 shows a cell with the small compact form of the Golgi apparatus (G). The cell to

the left of this represents an early stage of the enlargement of the apparatus, the more advanced stages being represented in figs. 3 and 4. In the latter figure especially are numerous secretion granules (*s*) closely associated with the apparatus. Identical pictures can be seen in good silver preparations by the Cajal or Da Fano methods. With none of the good cytoplasmic fixatives have I been able to see any evidence of a vacuolar system such as Parat's theory would imply. A series of experiments was carried out with the Dietrich-Parat method. Pieces of pancreas were fixed in Helly's fluid, and then transferred to a saturated solution of potassium bichromate kept at 35° C. Each day, for 5 days, pieces of tissue were removed, washed thoroughly and embedded. Sections were stained by the Volkonsky method (1928), which is as follows:—

1. Staining in Rubin S in aniline water (20 per cent. solution) on a hot plate.
2. Differentiation in aurantia 0.5 per cent. solution in 70 per cent. alcohol until mitochondria are visible.
3. Rapid washing.
4. Fixation of mitochondrial staining in: phosphomolybdic acid, 1 gr.; normal caustic soda, 10 c.c.; distilled water, 90 c.c.
5. Careful washing.
6. Staining in: methylene violet, 0.4 gr.; azure II, 0.1 gr.; potassium carbonate, 0.1 gr.; glycerine, 50 c.c.; distilled water, 50 c.c.
7. Washing in distilled water.
8. Differentiation in Unna's tannin orange mixture.

By this method mitochondria are stained red, the chromatin of the nucleus blue and the plasmosomes red. The demonstration of the mitochondria by this method depends upon the oxidisation of their lipin content so that it is no longer soluble in the re-agents employed for dehydrating and embedding.

The examination of the series of preparations made according to this technique showed no appreciable difference in cell structure after the tissue had been kept in saturated potassium bichromate for 2 days. Figs. 5 and 6 show two cells from the material which had remained in bichromate at 35° C. for 5 days. They show the typical filamentous mitochondria (*m*) which are visible in the living cell without any staining. In that part of the cytoplasm which is occupied in figs. 1 to 4 by the Golgi apparatus is seen a canalicular system (*c*). In some of my preparations its walls are stained faintly blue. Secretion droplets (*s*) are seen in contact with it. There is no doubt that this canalicular system and the blackened Golgi apparatus as shown in figs. 1 to 4

are one and the same thing. While it blackens with osmic acid its reaction to the Dietrich-Parat method for lipins is negative. The latter result does not necessarily imply that no lipins are present around the canalicular system. From the property of these substances to lower surface tension one would expect them to accumulate at the surface of such a canalicular system. The fact cannot be ignored, however, that the walls of the canaliculi may be composed of a lipid substance which is not demonstrable by the Ciaccio method and its modification, or that the lipid is present in such a fine film as to be below what Klein calls the "Erfassungsgrenze" for the tests. The structure in the living cells responsible for the Golgi apparatus in our impregnated preparations might be ultramicroscopic, but against this view should be considered the observations of Bensley (1911) and O'Leary (1930) that a canalicular system is visible in the living cells of the islets.

I have attempted to modify the impregnation of the Golgi apparatus by subjecting mice to ether anæsthesia for periods of 1 and 3 hours, then removing the pancreas and treating according to my modified Kopsch technique. No appreciable change was brought about by this treatment.

#### *4. Vital Staining of the Acinar Cells with Neutral Red ; and Cytological Technique for making Permanent Preparations of Vitrally Stained Tissues.*

Following the injection of neutral red into living mice, dye droplets appear in the acinar cells. I have not seen them appear when the pancreas is teased out in saline containing neutral red and examined under the microscope at room temperature. By this method secretion granules are ultimately stained. Such experiences suggest that the formation of the dye droplets is brought about by the vital activity of the cells and is not due to a passive staining of preformed droplets.

It is usually necessary for the cells of vitally stained animals to be examined in the fresh state, immediately after removal from the body. This is due to the great difficulty of retaining basic dyes. I have made numerous attempts to find a fixative which would enable neutral red to be retained in histological preparations. I have used as fixatives mercuric chloride, chromic acid, potassium bichromate, picric acid, osmic acid, platinum chloride, gold chloride, various mixtures of these like Champy's fluid, Mann's corrosive osmic, Bouin's fluid, Mitamura's (1923) fixatives, and formol-bichromate. I have employed the formol vapour method of Gros, quoted by von Möllendorff (1921) in which tissues are fixed in an atmosphere of water vapour saturated with formol. Golovine's (1902) method was also employed, the essential feature of which is

dehydrating in graded alcohols to which ammonium molybdate has been added, and finally in a saturated solution of picric acid in absolute alcohol. I have tried fixation in boiling solutions of sublimate and bichromate, and in the same solutions cooled with ice. Instead of using alcohol for dehydrating I have employed acetone. This medium with mercuric chloride dissolved in it has also been used as a fixative, followed by washing in pure acetone and clearing in benzene.

By none of these methods have I been able to make satisfactory permanent preparations of tissue vitally stained with neutral red. The only method with which I have had some measure of success is that of Gardner (1926-27). From the published account of this method it is difficult to know exactly how the fixative should be made. Gardner's instructions are to "add sufficient solid NaOH to U.S.P. formaldehyde solution to give a pH value of 7.6. Dissolve by gentle heating. Keep in the incubator at 37° C. Of the alkaline formaldehyde add 15 per cent. to the usual Zenker's fluid (without acetic acid)." When normal NaOH is added drop by drop to formol until the neutral point is reached every extra drop makes a marked difference unless a large quantity of formol has been used. As Gardner used solid NaOH, and found it necessary to employ heat to dissolve it, this final solution would appear to have been strongly alkaline. Since Gardner says "the sodium not only neutralises the acidity, but it has a specific mordanting effect upon staining," I considered it desirable to investigate the influence of varying the pH of the formol used in the fixative. I found that it was a disadvantage to use a fixative containing formol with a pH much above 7, since red dye droplets present in the tissue are changed to yellow and are difficult to distinguish. The best results I have obtained with a fixative consisting of:—

43 c.c. Zenker's fluid (without acetic acid),

7 c.c. of formol to which 2 drops of normal NaOH have been added.

After 24 hours, following Gardner's method, the fixed material is cut up into small blocks not more than 2 mm. in thickness and transferred to fresh Zenker's fluid (without acetic acid). It can be dehydrated immediately, or within 2 days. Before dehydration Gardner washes for a quarter of an hour, and then blots the small blocks of tissue. Then they are put into 80 per cent. alcohol for 10 minutes followed by graded mixtures of 95 per cent. alcohol and benzene in the following proportions:—9 : 1, 8 : 2, 7 : 3, 5 : 5, 3 : 7, 2 : 8, and 1 : 9, for 10 minutes in each. The clearing is done in benzene, one or more changes for an hour or two; the embedding in benzene saturated with wax at 37° C. for an hour, followed by paraffin at 56° C. (four changes in 30 minutes). Various

modifications of this method have been tried such as fixation at 0° C., adding neutral red solution to the fixative in various amounts and using the filtered solution, but none of these methods showed any advantage.

In successful preparations by this method the neutral red droplets are clearly distinguishable in unstained sections, but Gardner has devised a method of staining with Harris' hæmatoxylin, which it is possible to employ in certain cases.

Two cells from a permanent preparation by this method of the pancreas of a supravivally stained mouse are shown in figs. 7 and 8. The appearance of the dye droplets in the cells is identical with that in the living cells. The mouse from which these cells were taken had received subcutaneous injection of 0.5 c.c. of a 0.5 per cent. solution of neutral red in distilled water on the first, second and third days of the experiment. On the fourth day 1 c.c. of the same strength solution was injected, and the animals were killed 6½ hours afterwards. A similar staining result was obtained when tissues were examined 5 hours after a single subcutaneous injection of 1 c.c. of the dye.

The problem which such staining as that shown in figs. 7 and 8 (Plate 9) presents is whether the droplets were present before the dye was injected, or whether they represent an accumulation of dyestuff brought about by the vital activities of the cells. That the latter is the true explanation seems indicated by experiments which we shall next consider.

#### *5. The Osmic Impregnation of Vitrally Stained Acinar Cells.*

In order to ascertain what relationship existed between the dye droplets shown in figs. 7 and 8, and the canalicular system represented in figs. 1 to 6, preparations were made of the pancreas of vitally stained mice according to my modified Kopsch technique. For the purpose of the present thesis it will suffice to confine our attention to osmic impregnated preparations of the same mouse pancreas as that from which figs. 7 and 8 were drawn. In figs. 9, 10 and 11 are shown three pancreas cells of this mouse. The alteration brought about in the structure of the cell by vital staining is immediately obvious when these cells are compared with the unstained ones shown in figs. 1 to 4. The osmicated reticulate structure (G) in the latter is broken up in figs. 9 to 11. Amongst the strands occur large osmicated bodies (*d*). The appearance presented by these figures suggests that the dye droplets, such as are seen in figs. 7 and 8 have formed within the reticulate structure (G), have caused it to become dilated in places, and hence destroyed its continuity.

After the investigations described in this paper had been completed a paper

was published by Beams (1930) in which he compares the vitally stained acinar cells of the rat pancreas with osmic impregnated preparations of the same. Beams says his preparations disclose "the vacuome and the Golgi apparatus side by side in the same cell. The vacuome may be embedded among the strands of the Golgi apparatus, but never fused with them." He concludes that "the Golgi apparatus and the vacuome are two discrete substances in the acinar cells of the pancreas, both of which may occupy the same general position in the cell." While I am able to confirm Beams' main thesis that the Golgi apparatus does not arise by a fusion of the neutral red granules, my observations do not accord with his view that the vacuome is never fused with the Golgi apparatus. It is true that dye droplets sometimes occur in the acinar cells after vital staining, which are remote from the Golgi apparatus, and bear no obvious relationship to it. For the most part I believe that the evidence is in favour of the view that the dye droplets originate either within the canalicular system as Alexenko (1929) describes for nerve cells, or else are formed in direct contact with it. Further evidence for this view is afforded by the study of the acinar cells after staining over a longer period of time.

Figs. 15 and 16 (Plate 9) show cells from a tumour-bearing mouse which had received daily injections of 0.5 c.c. of an 0.5 per cent. solution of neutral red during 5 days. It was killed 48 hours after the last injection owing to its poor condition. *Post-mortem* examination showed considerable cedema beneath the skin, and within the abdominal cavity. The internal organs had lost most of their colouration and appeared a very pale pink colour. The most conspicuous feature of osmic preparations of the pancreas was the greatly enlarged Golgi apparatus as is seen in the figs. 15 and 16 (Plate 9). Another peculiarity was the relatively small amount of zymogen granules in the cells. Portions of the same gland which had been fixed in Helly's fluid alone, and others which had been treated according to the Dietrich-Parat method showed the same canalicular structure in the acinar cells. The preparation gave no indication that the canalicular structure had arisen by coalescence of droplets. They suggested rather that there had been brought about a blockage of this system owing to the accumulation of the dyestuff, or of products of its metabolism, which has prevented the elaboration of zymogen granules.

#### 6. *Changes in Cell Structure during Autolysis.*

I thought it probable at first that the enlargement of the Golgi apparatus as seen in figs. 15 and 16 might be due to a degeneration of the acinar cells in a sick animal. To find whether this was so normal mice were killed and placed

in an ice safe, and the pancreas then removed and treated by my modification of the Kopsch technique at intervals of 24, 50 and 75 hours. At none of the stages examined did I find any indication of an enlargement or swelling up of the Golgi apparatus. Fig. 12 shows an acinar cell drawn from a preparation of the pancreas fixed 50 hours after death. It represents one of the least altered cells at this stage, and is typical of the earliest phase of disintegration of the cell structure. The Golgi apparatus (G) is here seen broken up into droplets some larger than others, and resembling fat droplets. Such a fragmentation is the characteristic change that occurs during autolysis. At more advanced stages it proceeds to such an extent that no indication remains of any reticulate structure.

*7. The Difference between Vital Staining of the Acinar Cells with Neutral Red and with Rhodamin B.*

According to von Möllendorff's conception neutral red is an example of a basic dye which is only slightly soluble in lipins, but is readily flocculated in contact with acid colloids; while rhodamin B is readily soluble in lipins, but is not flocculated in contact with acid colloids. After vital staining with neutral red dye droplets appear in the cells as has been already described, while rhodamin produces just a diffuse staining of the cells. From Chlopin's work we know, that following vital staining with neutral red, granules can be demonstrated in sections when they are stained with certain basic dyes (Krinom). In the acinar cells of the pancreas of the axolotyl he has figured these granules arranged in such a way as to resemble in a most striking manner the Golgi apparatus. Chlopin has pointed out, however, that this similarity between the two does not always exist. By following Chlopin's technique I have been able to demonstrate for the first time the presence of the "Krinom" in mammalian cells. After a single injection of 1 c.c. of a 0.5 per cent. solution of neutral red into a mouse I found no evidence of the "Krinom" in the acinar cells. In the cells of the same mouse as that from which figs. 7 to 11 were drawn it was readily demonstrable after Helly fixation followed by cois-azure staining. The mice of the series to which this one belonged had received a subcutaneous injection of 0.5 c.c. of a 0.5 per cent. solution of neutral red daily, for 3 days, and 1 c.c. of the same solution the fourth day. It was killed 6½ hours after the last injection. In fig. 13 the granules (K) constituting the "Krinom" occupy a position comparable with that of the Golgi apparatus in fig. 2, while in fig. 14 they are arranged in a typical reticulate fashion. After vital staining with the same strength solution of rhodamin B, and with the



same daily injection, over a longer period of time, no such granules could be demonstrated in fixed preparations made by the same method. Such granulations were also absent from control preparations. The presence of granules demonstrable by Chlopin's method affords indisputable evidence that in vital staining with neutral red new formations arise in the cells.

#### 8. *The Different Permeability of Acinar Cells of the Pancreas to Acid and Basic Dyes.*

Although the acinar cells of the pancreas stain readily with basic dyes they remain uncoloured when animals are vitally stained with acid dyes. I have examined the pancreas of mice after intense intra vitam staining with trypan blue and carmine. Although the cells of the inter-alveolar connective tissue stain deeply, the acinar cells always remain uncoloured. This difference would seem to be due to the impermeability of the acinar cells to acid dyestuffs.

#### 9. *Summary and Conclusions.*

The result of the experimental study of the acinar cells of the mouse pancreas described in this paper may be summarised as follows :—

1. In cytological preparations which demonstrate mitochondria there can be seen a canalicular system (figs. 5 and 6). Osmic and silver impregnation methods show this as a black network—the Golgi apparatus (figs. 1 to 4). Secretion granules arise in intimate relationship with this network. Impregnation of it by the osmic technique is not influenced by ether anaesthesia extending over 3 hours.

2. After intra vital staining with neutral red, dye droplets appear in the cells, which can be retained in permanent preparations (figs. 7 and 8). For the most part these dye droplets are localised in that part of the cytoplasm where in other preparations is seen the canalicular system or Golgi apparatus.

3. After intra vitam staining with neutral red the Golgi apparatus is considerably altered. (Figs. 9 to 11, 15 and 16.)

4. The changes undergone by the Golgi apparatus during cell autolysis differ from those figured as characteristic of vital staining. During autolysis the apparatus merely becomes fragmented and fat droplets may appear amongst its strands (fig. 12).

5. Intense intra-vital staining with neutral red leads to the formation of deposits within the cell which can be stained in permanent preparations by the eosin-azure method (figs. 13 and 14). Such granules are not formed after intense staining with the diffuse-staining dyestuff rhodamin B.

On the basis of these observations and in the light of the work of other investigators previously discussed, it is concluded :—

1. There is no fundamental difference between vital staining with an acid dye, such as trypan blue ; and a basic dye, such as neutral red. Both give rise to new formations in the cell, and both under certain conditions can stain preformed structures, such staining being determined by the physico-chemical properties of the bodies stained. Many types of cells, which are permeable to basic dyes are, however, impermeable to acid dyes.

2. There is present in the acinar cells of the pancreas a reticulate structure, or canalicular system, which is distinct from the mitochondria. When such cells are stained intra-vitally with neutral red, dye droplets are formed, either within the strands of the reticulate structure, or else in direct contact with it.

#### 10. BIBLIOGRAPHY.

- Alexenko, B. (1929). 'Mem. Acad. Sci. Ukraine,' vol. 12, p. 173.  
 Beams, H. W. (1930). 'Anat. Rec.,' vol. 45, p. 137.  
 Bensley, R. R. (1911). 'Am. J. Anat.,' vol. 12, p. 297.  
 Chlopin, N. G. (1927). 'Arch. expt. Zellforsch.,' vol. 4, p. 462.  
 Covell, W. P., and Scott, G. H. (1928). 'Anat. Rec.,' vol. 38, p. 377.  
 Cowdry, E. V. (1918). "Contrib. to Embry." Carnegie Inst., Washington, vol. 8, p. 39.  
 Cowdry, E. V., and Scott, G. H. (1928). 'Arch. Inst. Pasteur Tunis,' vol. 17, p. 233.  
 Feyel, P. (1928). 'Arch. anat. microsc.,' vol. 24, p. 359.  
 Gardner, L. U. (1926-27). 'Proc. Soc. Expt. Biol. and Med.,' vol. 24, p. 646.  
 Gatenby, J. Brontë, and Nath, V. (1926). 'Q. J. M. S.,' vol. 70, p. 371.  
 Gatenby, J. B. (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 302.  
 Gatenby, J. B., and Wigoder, S. (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 351.  
 Giroud, A. (1929). 'Protoplasma,' vol. 7, p. 72.  
 Golovine, E. (1902). 'Z. wiss. Mikr.,' vol. 19, p. 176.  
 Heldenhain, M. (1908). 'Handb. Anat. Menschen, Jena.'  
 Hirschler, J. (1928a). 'Z. Zellforsch.,' vol. 7, p. 62.  
 Hirschler, J., and Monné, L. (1928b). 'Z. Zellforsch.,' vol. 7, p. 201.  
 Krjukowa, Z. I. (1929). 'Arch. Russes Anat. Hist. Embry.,' vol. 8, p. 25.  
 Ludford, R. J. (1926). 'J. Roy. Mic. Soc.,' p. 107.  
 Ludford, R. J. (1927). 'Proc. Roy. Soc.,' B, vol. 101, p. 409.  
 Ludford, R. J. (1928). 'Proc. Roy. Soc.,' B, vol. 103, p. 288.  
 Ludford, R. J. (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 493.  
 Ma, Wen-Chao, and Chang, Hsi-Chun (1928). 'Chinese J. of Physiol.,' vol. 2, p. 381.  
 Ma, Wen-Chao, Chang, Hsi-Chun, and Liu, An-Ch'ang (1929). 'Chinese J. of Physiol.,' vol. 3, p. 29.  
 Mitamura, T. (1923). 'Zentralblatt allgem. Path. path. Anat.,' vol. 33, p. 593.  
 v. Möllendorff, W. (1920). 'Ergeb. Physiol.,' vol. 18, p. 141.  
 v. Möllendorff, W. (1921). 'Abderhalden's Hdb. biol. Arbeitsmeth.,' Sect. 5, Pt. 2, p. 97.  
 O'Leary, J. L. O. (1930). 'Anat. Rec.,' vol. 45, p. 27.  
 Parat, M., and Painlevé, J. (1925). 'Bull. Hist. Appl.,' vol. 2, p. 1.

- Esch, M. (1928). 'Arch. anat. mic.,' vol. 24, p. 73.  
 Teichmann (1891). "Mikroskopischer Beitrag zur Lehre der Fettresorption," 'Inaug. Diss. (quoted by v. Mollendorff, 1920).  
 Volkonaky, M. (1928). 'Bull. Histol.,' vol. 5, p. 220.  
 Weiner, P. (1930). 'Z. mikr. anat. Forschung.,' vol. 20, p. 122.  
 Zweibaum, J., and Elkner, A. (1930). 'Arch. expt. Zellforsch.,' vol. 9, p. 419.

## 11. DESCRIPTION OF PLATE 9.

All the figures represent acinar cells from the pancreas of the mouse.

### DESCRIPTION OF LETTERING.

*c*, canalicular system, or negative Golgi apparatus. *d*, dye droplets after intra vital staining with neutral red. *f*, fat droplet. *G*, Golgi apparatus, or osmic impregnated canalicular system. *k*, "Krinom" of Chlopin, or granules stainable with basic dyes after intra vital staining with neutral red. *m*, mitochondria. *s*, zymogen granules.

Figs. 1-4 show the relation between the Golgi apparatus (*G*) and products of secretory activity, the zymogen granules (*s*). (Modified Kopsch method.)

Figs. 5 and 6 show the canalicular system (*c*) or negative Golgi apparatus. Zymogen granules (*s*) are seen amongst its strands. Mitochondria (*m*) are quite distinct from the canalicular system (*c*). (Dietrich-Parat-Volkonaky method.)

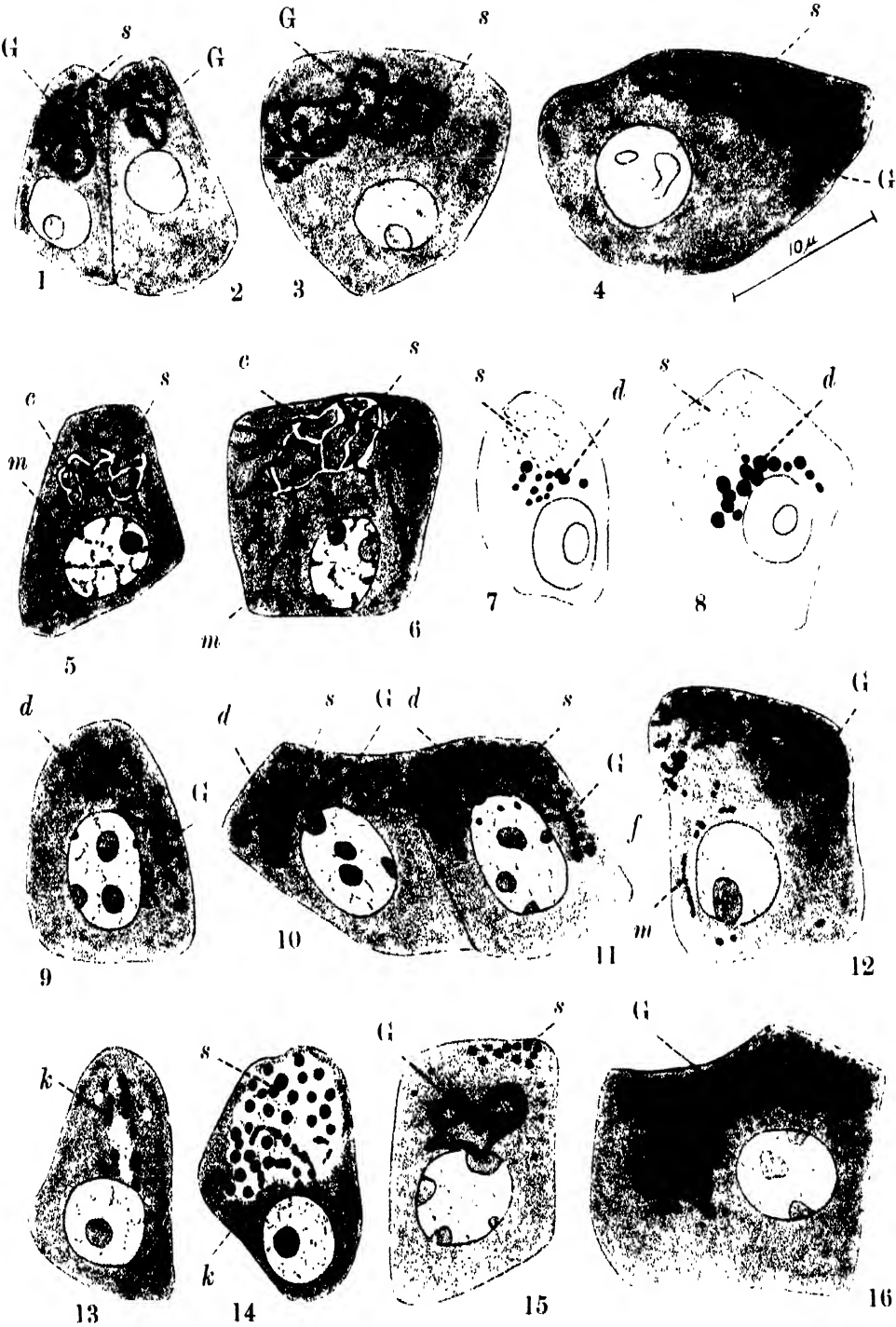
Figs. 7 and 8 are cells drawn from a permanent preparation of the pancreas, intravitaly stained with neutral red, and show the dye droplets (*d*). (Gardner's method, section unstained.)

Figs. 9-11 show the distortion of the Golgi apparatus (*G*) brought about by intra-vitam staining with neutral red. The dye droplets (*d*), impregnated by osmic acid, occur amongst the strands of the Golgi apparatus. (Modified Kopsch technique.) Protocol of experiment: 0.5 c.c. of 0.5 per cent. solution of neutral red injected subcutaneously on the first, second, and third days of the experiment; 1 c.c. on the fourth day, and the mouse killed 6½ hours later.

FIG. 12. One of the least altered cells from a mouse which had been kept 50 hours after death in an ice safe. The Golgi apparatus (*G*) is broken up into granules or droplets amongst which occur some fat globules (*f*). (Modified Kopsch technique.)

Figs. 13 and 14 show the presence of deposits (*k*) in the cells formed as the result of vital staining. These granules (*k*) called the "Krinom" by Chlopin (1927) resemble the Golgi apparatus in their general arrangement and distribution in the cell. (Holly fixation, followed by Eosin-Azure staining.)

Figs. 15 and 16 show an apparent hypertrophy of the Golgi apparatus subsequent to intra vital staining with neutral red. (Modified Kopsch technique.) Protocol of experiment: 0.5 c.c. of 0.5 per cent. solution of neutral red injected subcutaneously on the first, second, third, fourth and fifth days of the experiment, and the mouse killed 48 hours later.





*The Heat Production in Isometric and Isotonic Twitches.*

By A. V. HILL, F.R.S., Foulerton Research Professor, University College, London.

(Received July 26, 1930.)

In a recent paper from Meyerhof's laboratory P. Rothschild (1930) has shown that in a series of isometric twitches of a frog's gastrocnemius or semi-membranosus considerably more lactic acid may be liberated than in the same number of isotonic twitches with the same initial load: while with sartorius there may be little or no difference. 90 to 150 shocks were applied, at intervals of 5 to 10 seconds, at room temperature (11° C. to 17° C.), and either directly or to the nerve: the intervals were sufficient to allow complete relaxation between twitches and the number of twitches was not so great as to cause appreciable fatigue. With semi-membranosus, with an initial load of 10 or 20 g. the lactic acid in the isotonic averaged about 35 per cent. less than in the isometric twitches: with gastrocnemius stimulated through its nerve the following six results were obtained at 15° to 17° C. in 92 to 102 twitches:

Initial load: g. ....	25	20	20	20	10	10
After-load (isotonic): g. ....	13	50	50	50	140	140
Deficit due to shortening: p.c. ....	70	49	64	76	34	25

Thus in an isotonic contraction with considerable shortening the lactic acid may be  $\frac{1}{2}$  to  $\frac{1}{3}$  of the amount in an isometric contraction with the same initial load: while when the shortening is smaller (greater after-load) the difference may be less (last two experiments). With sartorius, however, in 110 to 150 contractions at 12° C. to 17° C., there was a slight *excess* of lactic acid in the isotonic contractions, averaging about 8 per cent. The smallness of the difference was regarded by Rothschild as insignificant, in view of the probable error of his estimations: in this, as will be seen, he unduly depreciated the accuracy of his own observations: his difference was probably genuine.

With gastrocnemius and semi-membranosus the difference observed by Rothschild is so large that it cannot possibly be doubted. It is, however, the direct opposite of that obtained by Fenn (1923) with sartorius, according to whom "whenever a muscle shortens upon stimulation and does work in lifting a weight, an extra amount of energy is mobilised which does not appear in an isometric contraction." Fenn found this to be the case both for twitches and

for short tetanic contractions. Rothschild's results with gastrocnemius and semi-membranosus are also in disagreement with those of Hartree and Hill (1928) with sartorius, who found "in a single twitch the total energy set free is the same whether work (on the Levin-Wyman ergometer) be done or not." So far as they went Rothschild's five experiments with sartorius giving a slight excess of lactic acid production in isotonic twitches, might be regarded as consistent with Fenn's results, or, alternatively, if the 8 per cent. difference could be disregarded, as agreeing with the conclusions of Hartree and Hill.

It would be a peculiar thing if, as Meyerhof suggests (1930, p. 255), sartorius proved to be a special case among muscles. It is indeed almost inconceivable that the fibres of sartorius should differ from those of gastrocnemius in respect of such a fundamental property. The difference between sartorius and gastrocnemius cannot be one of the mechanism of muscular response: it must lie in the distribution of the fibres, and in the mechanical properties of the muscle, with its connective tissue, regarded as a whole. Sartorius is very extensible, gastrocnemius very inextensible, semi-membranosus intermediate between the two. There was no reason to disbelieve any of the observations referred to: their apparent disagreement must be due to the neglect of some unsuspected factor. This factor has been found: it is the length of the muscle. All muscles, with a sufficiently small initial load, show the effect described by Rothschild; all muscles, with a sufficiently great initial load, show the effect described by Fenn; and over an intermediate range of loads there is approximate equality, as found by Hartree and Hill.

The reason for the apparent difference between the three muscles is simply that sartorius is greatly extended by quite small loads while the others are not. Under a load of 5 g. the fibres of sartorius may be as much extended as those of gastrocnemius under a load of 100 g.: in the latter case the weight is held by connective tissue in parallel with the fibres. To observe with sartorius the effect described by Rothschild it is necessary to work with initial loads of 1 or 2 g.; to observe with semi-membranosus the effect described by Fenn it is necessary to work usually with great initial loads. This statement is true for single twitches. For tetanic contractions the region of loads within which the isotonic twitch liberates less energy than the isometric, shrinks and may almost disappear. The recognition that the "Fenn effect" is limited to a certain range of lengths and loads will require a reinvestigation of the whole problem. This paper deals mainly with single twitches, and with no forms of contraction other than isometric and isotonic.

*Methods.*—Experiments have been made either at room temperature (15° to

21° C.), or at 0° C., on Hungarian esculenta or English temporaria frogs. The thermopiles employed have been either of the "all metal" type commonly used in this laboratory, or of a new rapid type ("Q") recently designed and constructed by Mr. A. C. Downing. Being wound on brass or silver frames they effectively prevent differences of temperature from persisting in the instruments, which is important when the muscles are allowed to shorten considerably. The galvanometer was a very sensitive moving coil (Zc) by Kipp. A single maximal twitch gave deflections from 100 to 500 mm. on the scale, depending on conditions, readable if required to a fraction of 1 mm. Stimulating electrodes were so arranged that they exerted little or no constraint or friction on the muscles. Single break shocks were supplied by a Harvard coil. The muscles were connected by a thick linen thread to a lever carried on a Palmer stand. The lever bore a load near its axis and its end rested on a stop. The stand was screwed up so that the load was just, and only just, lifted: in this way changes of length of the muscles could be read to 0.1 mm. For isometric contractions the lever was merely clamped rigidly to the stop. For quick releases a magnet was employed exactly similar to that described by Gasser and Hill (1924, fig. 1, p. 401): the moment of release was determined by a Lucas revolving contact breaker (see Parkinson, 1926). The lever wrote upon a glass plate held in a clamp. Positive "after loads" were given in the usual way by extra weights hung upon the supported lever. Negative after loads were given by connecting the lever (after exact adjustment on to the stop for the initial load) to the quick release mechanism, then removing some of the initial load, and releasing the muscle immediately after the stimulus. All stimulation was direct.

Calibration was as usual: when a comparison was required of the heat production at different lengths, calibrations were made at several lengths, the results plotted and the numbers required obtained by interpolation.

When a muscle has been stretched by a considerable load it does not return to its original length under a smaller load without certain precautions. In a series with diminishing loads it is necessary either to give two or three shocks at a given load in order to obtain adjustment to that load: this may cause unnecessary fatigue: or—better—to release completely between stimuli, and then load as required half a minute before the next shock. In most cases observations have been made first with increasing loads (isotonic and isometric at each length), and then with decreasing loads: by releasing the muscle between stimuli in the reverse series very consistent results were obtained.

In most cases a pair of muscles was employed: when only one was used the



actual load in grammes has been doubled in the figures to represent the case of a pair.

### Results.

*Semi-membranosus*.—The results are most clearly shown in diagrams. Fig. 1 represents a case at 16° C. in which the heat in isotonic twitches was less

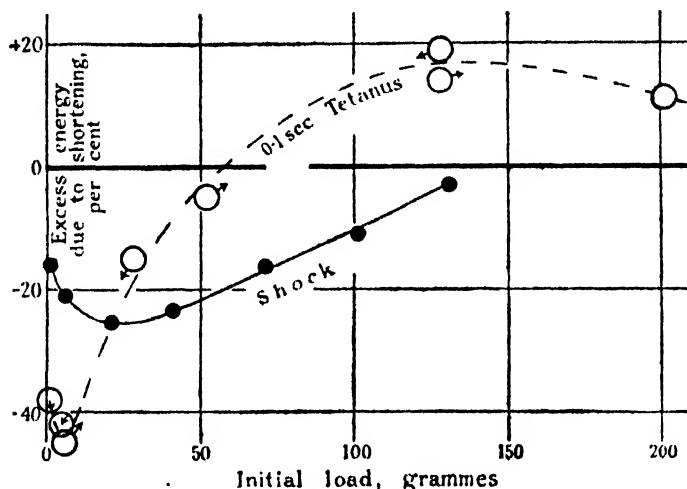


FIG. 1.—*Semi-membranosus* of *R. esc.* 19.5.30. About 16° C. Single shocks and 0.1 second tetanus. Excess energy due to shortening plotted as a percentage, i.e., as 100 (isometric–isotonic)/isometric, against initial load. Arrows on the tetanus curve show the sequence of observations, starting on the left.

than in isometric up to rather great initial loads. For comparison the results of a 0.1 second tetanus are given in the same figure. Here we see both phases, and the region of loads within which the isotonic heat is less is closing up. On the next day an exactly similar experiment was performed. At 200

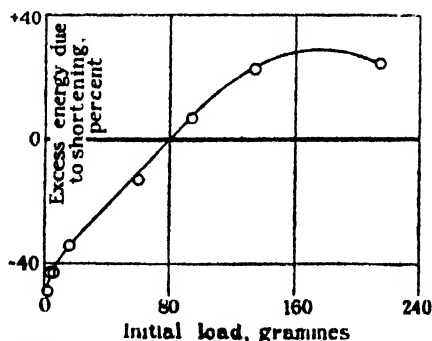


FIG. 2.—*Semi-membranosus* of *R. esc.* 27.5.30. About 15° C. Single shocks. Good reverse.

grammes initial load, in a twitch, the isotonic heat was still just less; in a 0.1 second tetanus, however, the isotonic heat was greater beyond 66 grammes. In some muscles, however, particularly at 0° C., the curve crosses the zero line rather early, even in the case of twitches. In fig. 2 is an example at 15° C.: an excellent experiment with good reverse series, means shown in the figure. In fig. 3 are two examples at 0° C.: A was on an English *R. temp.*, fresh, B on a Hungarian *R. esc.*, after 18 hours at 0° C. in Ringer's solution.

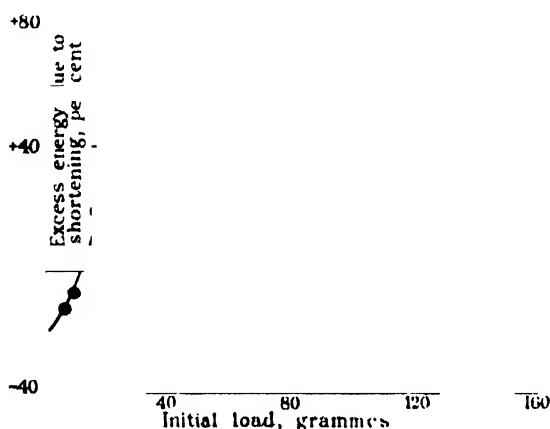


FIG. 3.—Semi-membranosus of *R. temp.* 10.7.30 (A), of *R. esc.*, 18.6.30 (B). At 0° C. Single shocks. Muscle (B) had been used for an experiment at 20° C. on 17.6.30, and was kept all night in Ringer's solution at 0° C., and used at 0° C. next day. It was in good condition.

*Sartorius*.—That the same phenomena occur with sartorius is well shown by the following experiment.

Experiment of 15.5.30.—Sartorii of *R. esc.* at 15.5° C. after 16 hours in oxygenated Ringer's solution. Single shocks.

(A) load on muscles 1 gramme: "extension" 0 mm. Galvanometer deflections: mm.

Isotonic .....	84	86	85	84	84	84	mean 84½
Isometric .....	135	130	132	131	130		mean 132

Excess due to shortening, — 36 per cent.

(B) Load on muscles 21 grammes: "extension" 5.3 mm.

Isotonic .....	119	116	118	116		mean 117
Isometric .....	117	115	114			mean 115

Excess due to shortening, + 2 per cent.

(C) Load on muscles 41 grammes: "extension" 7.1 mm.

Isotonic .....	121	120	123	123		mean 122
Isometric .....	106	106	105			mean 106

Excess due to shortening, + 15 per cent.

With 20 grammes initial load, 10 grammes on each muscle, isotonic and isometric heats were almost exactly equal: above this load isotonic heat was

the greater, below it isometric. In fig. 4 a curve is shown for  $18^{\circ}\text{C}$ . up to 83 grammes initial load : it crosses the zero line at 18 grammes (9 grammes

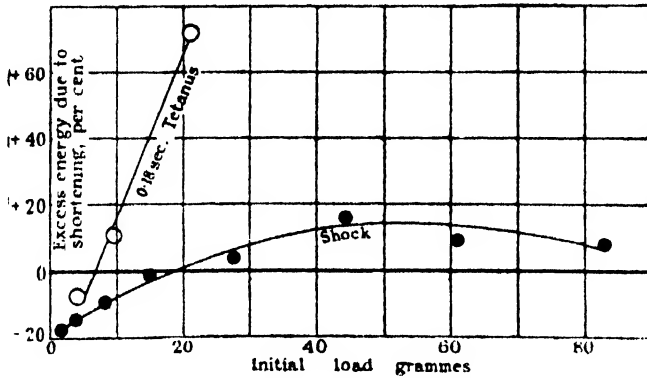


FIG. 4.—Sartorius of *R. esc.* 2.6.30. At  $18^{\circ}\text{C}$ . Single shocks and 0.18 second tetanus. Good reverse in each case.

on each muscle). For comparison, the beginning of the curve for 0.18 second tetanus is given : as in fig. 1 it has shifted considerably to the left and risen higher. In a similar experiment at  $16^{\circ}\text{C}$ . the curve for single shocks crossed at 12 grammes : for 0.1 second tetanus it crossed at 5 grammes, and rose several times as far. In the experiment of 15th May, 1930, described above, in a 0.1 second tetanus the isotonic heat was 29 per cent. less than the isometric heat at 1 gramme load, equal to it at 6 grammes load, and 60 per cent. greater at 25 grammes load.

The curve is not necessarily the same with different muscles, nor even for the same muscle at different times. In fig. 5, for example, at  $0^{\circ}\text{C}$ ., the curve

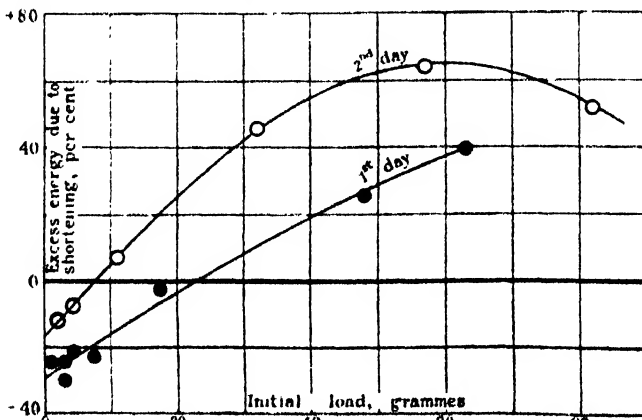


FIG. 5.—Sartorius of *R. esc.* 24-25.6.30. Single shocks at  $0^{\circ}\text{C}$ . Observations on second day after night in oxygenated Ringer's solution at  $0^{\circ}\text{C}$ .

taken on the second day is very different from that on the first, although the muscle was apparently in perfect condition on both occasions. Usually the negative phase of the curve with small initial loads is very obvious, *e.g.*, in the case given above and in figs. 4 and 5: the positive phase is always found at great initial loads. Rarely the negative phase of the curve is absent, as seen in fig. 8 below.

Presumably Fenn, so far as he employed single twitches, always worked in the region of initial loads where the isotonic heat is greater than the isometric. It is quite easy to do so: the loads given here refer mostly to large Hungarian frogs: with English frogs a few grammes would cover the whole range of smaller isotonic heats. When he used tetanic stimuli it would have been difficult to avoid working in the region of greater isotonic heats. Hartree and Hill never compared simple isotonic and isometric twitches. They used the Levin-Wyman ergometer, often with limited shortenings and a certain amount of delay in release, together with a light isotonic lever to pull the muscle back to its initial position. The load on this lever, it now seems, must always have been rather smaller than Fenn's and near that at which isometric and isotonic heats are equal. Furthermore, their limited shortenings, their delay in release, and the fact that against the ergometer movement is much slower than against a lightly loaded isotonic lever, all would tend (as will be shown below) to make the total energy in working and in isometric contractions approximate to the same value. Rothschild's small excess of lactic acid in isotonic twitches simply means that he was working just beyond the point where the curve crosses the zero line.

The much wider range of loads with semi-membranosus within which the isotonic heat is less is probably due simply to a structural difference. This muscle, and still more gastrocnemius, is much less extensible than sartorius, which means presumably that the load is taken less by the fibres and more by connective tissue. If the muscle fibres are normally held in a state of extreme relaxation by the connective tissue, and only appreciably stretched under relatively great loads, we can understand why the region of smaller isotonic heats is so greatly extended.

*The relation between heat production and length.* - For isometric contractions this relation has been fully discussed by Hill (1925). For the present purpose it was necessary to investigate it specifically for single twitches, and for isotonic as well as isometric conditions. Fig. 6 shows an experiment on semi-membranosus at 15° C., the total energy being given for isometric and isotonic twitches. [The work done in an isotonic contraction appears as heat when

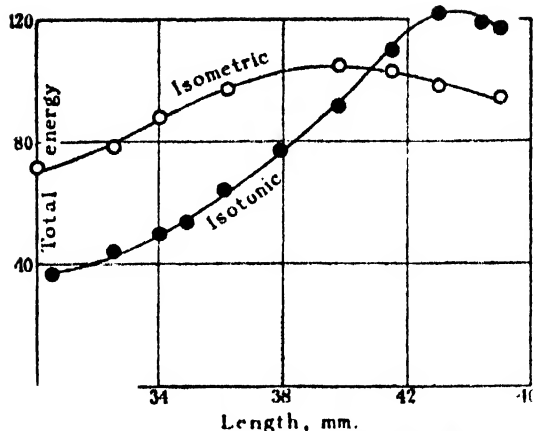


FIG. 6.—Semi-membranosus of *R. esc.* 27.5.30. Single shocks at 15° C. Total energy, arbitrary units, but corrected for length by calibration. Mean values of 4 series, isotonic →, isometric ←, isometric →, isotonic ←.

the load falls in relaxation, so the total energy is read on the galvanometer scale.] Fig. 7 shows a similar experiment on sartorius at 0° C. In this the

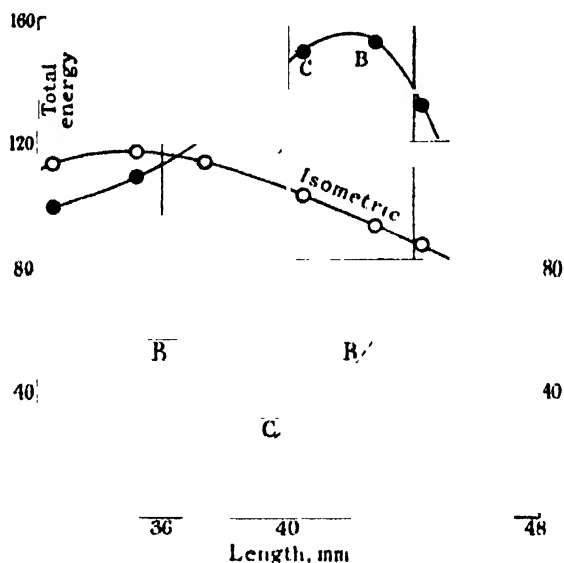


FIG. 7.—Sartorius of *R. esc.* 24.6.30. After night in oxygenated Ringer's solution at 0° C. Single shocks at 0° C. Total energy in arbitrary units but corrected for length by calibration: isotonic and isometric. Below (broken line) tension-length curve of resting muscle, together with three rectangles representing in load and amount of shortening the three isotonic contractions A, B and C.

tension-length curve of the resting muscle also is given, together with three rectangles to represent the isotonic twitches A, B and C. Fig. 8 represents

perhaps the best and most consistent experiment performed, but exceptional in that even at the smallest load (1 gramme) the isotonic heat was still

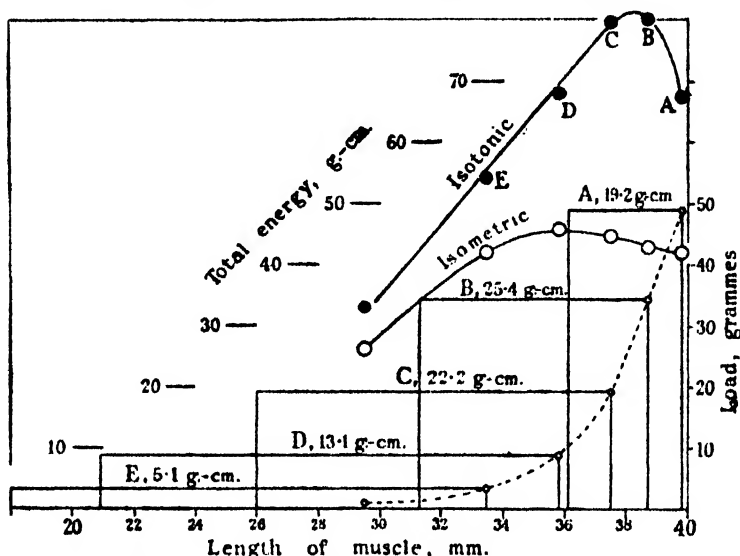


FIG. 8.—Sartorius of *R. esc.* 4.7.30. Single shocks at 0° C. Total energy in gramme-cm. isotonic and isometric. Series made, isotonic and isometric at each load →, then isotonic and isometric at each load ←. Means taken. Below (broken line) tension-length curve of resting muscle, together with 5 rectangles representing in load and amount of shortening the 5 isotonic contractions A, B, C, D and E. Over each rectangle the figure represents the gross work done in the twitch.

appreciably greater than the isometric. Here again the tension-length curve of the resting muscle is given, together with five rectangles to represent the isotonic twitches A, B, C, D and E. These rectangles and their meaning will be referred to later.

In some experiments, particularly in the first few performed, the isotonic heat never rose, at any length, above the maximum value of the isometric heat. This suggested a simple explanation of the excess or deficit of isotonic heat above or below isometric. Assuming (a) that heat production depends upon length, having a maximum value at some particular length, and (b) that length during contraction, as well as at the moment of stimulus, affects the liberation of energy, then (i) if shortening brings the muscle to lengths at which heat production is greater, isotonic heat will be greater than isometric, and (ii) conversely, if shortening brings the muscle to lengths at which heat production is less, then isotonic heat will be less than isometric. Assuming isometric heat to pass through a maximum at some particular initial load, we should expect

isotonic heat to be the greater at greater loads than this, and the less at smaller loads. In a general way all the curves show this kind of effect. I have no doubt that this is one of the factors involved. It is not, however, the only one, as is proved by figs. 6, 7 and 8, which show the more usual result. In all of these the isotonic heat at the greater loads is greater (in fig. 8 far greater) than the *maximum* value of the isometric heat. In contraction B, fig. 8, for example, the isotonic heat is 80 gramme-cm.; the maximum isometric heat is  $45\frac{1}{2}$  gramme-cm.; the average isometric heat over the length range of isotonic contraction B is about  $42\frac{1}{2}$  gramme-cm. It seems that the process of shortening against a load helps also to determine the energy set free.

It is natural to enquire whether, in such curves as those of figs. 6, 7 and 8, the excess energy bears any simple relation to the work performed. Various attempts have been made to find such a relation for the case of single twitches, but with little success. The matter is complicated by a factor seen in figs. 7 and 8, viz., the relatively large part of the work which is contributed, at the greater loads, by the elastic properties of the stretched muscle. If (i) the load were borne by inactive material arranged in parallel with the active fibres (or fibrillæ), and if (ii) this inactive material were supposed to have such a low "viscosity" as to yield up its whole potential energy when it shortened in a twitch, then we could allow for the error in question by subtracting from the gross work the area of the tension-length curve between the limits of the shortening. The net work would be the area of the rectangle above the broken line of the tension-length curve. Both assumptions, however, would be doubtful, the second indeed almost certainly untrue. The work done by the muscle, in virtue of its activity, is less than the gross work but greater than the net work so calculated by an indeterminate amount. No simple relation, therefore, between (a) work and (b) energy in excess of isometric, is likely to be found in such experiments as these. In a general way, however, it is evident that there are two factors involved: (a) an increase of total energy with work done and (b) a dependence of energy liberated on muscle length.

*Limited Contraction.*—During the experiment shown in fig. 2 a series of observations was made on a pair of muscles loaded with  $2\frac{1}{2}$  grammes, in which the amount of shortening was limited by a stop. With this load the free isotonic twitch gave 46 per cent. less heat than the isometric. The results are given in fig. 9. In this case it is clear that heat was determined by length and not by work, the less the shortening the more the heat. A similar experiment was performed on another muscle with the same result.

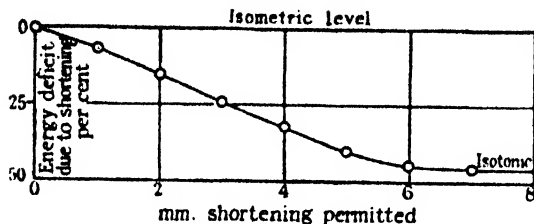


FIG. 9.—Semi-membranosus of *R. esc.* 27.5.30. About 15° C. Single shocks (exp. of fig. 2). Contractions of limited extent under 2½ grammes load.

*After-loading.*—Two cases of after-loading have been dealt with, (A) when the initial load is small and isotonic heat is less than isometric, and (B) when the initial load is greater and isotonic heat is more than isometric. An example of (A) is given in fig. 10. Here the free isotonic twitch under 2½ grammes

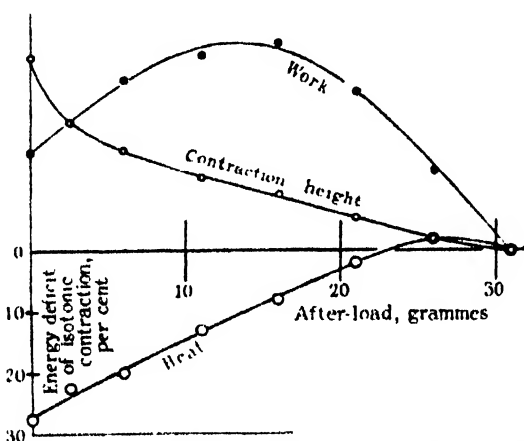


FIG. 10.—Sartorius of *R. esc.* 30.5.30. About 16° C. Single shocks. After-loaded. Initial load 2.5 grammes. Very good reverse.

initial load gave 27½ per cent. less heat than the isometric. With increasing after-loads the heat increased and the height of contraction diminished. There is an obvious inverse relationship between heat and contraction height. The work, however, increased to a maximum and then decreased to zero: the heat showed no parallel change. There is just a hint of a relation here between heat and work in the fact that the heat curve rises just above the isometric level for large after-loads; on the whole, however, fig. 10 can be regarded as illustrating the relation between heat and height of contraction, or length, and not that between heat and work.

An example of (B) is given in fig. 11. The observations recorded were made



during the experiment shown in fig. 8. The initial load was 19.3 grammes, corresponding to contraction C in that figure. In that contraction the elastic

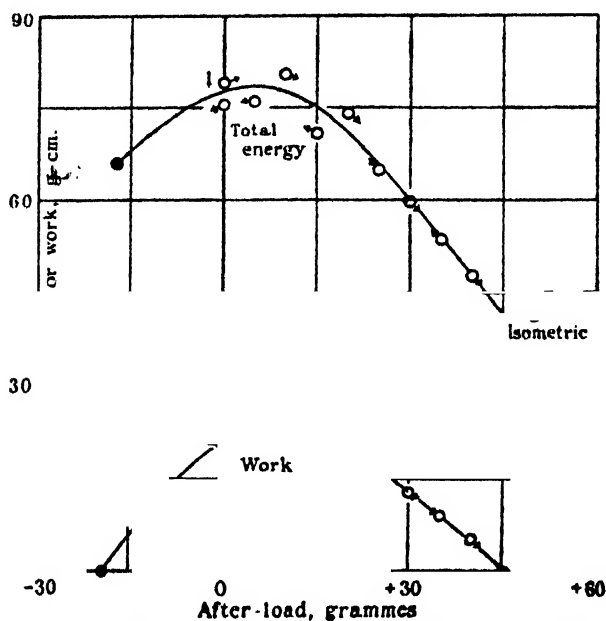


FIG. 11.—Sartorius of *R. esc.* 4.7.30. Same muscle as fig. 8. Single shocks at 0° C. Various positive and negative after-loads. Initial load 19.3 grammes. The full circles on the work curve are, right isometric zero, left unloaded zero. The full circles on the heat curve are, right mean isometric, left — 17 grammes after-load (raised by 2 gramme-cm. to allow for gradual loss of excitability). After the first isometric twitch observations began with the twitch marked 1 and proceeded as shown by arrows. At the end another isometric twitch was given, and finally the observation was made with the negative after-load.

work represented by the area of the tension-length curve was a fairly small fraction of the whole, allowing for "viscosity" or "hysteresis" in an actual twitch probably quite a small fraction. The work done, therefore, has a fairly definite meaning, which it would not have for a much greater load. The experiment started with an isometric twitch, followed by observation 1 with zero after-load. Readings were then made with a series of different after-loads, in the order of the arrows. The mean of the isometric heats is shown by the black circle to the right. Corresponding points for the gross work are shown below. There is a very clear parallelism between work and heat. To make this parallelism clearer the curves were continued to the left as follows. With a negative after-load of — 19.3 grammes (*i.e.*, a total load of zero during contraction) no work can be done. The work curve therefore must fall to zero

at  $-19.3$  grammes however far the muscle shortens: the full circle at the left end of the base line represents this point. A point at  $-17$  grammes after-load on the heat curve was obtained as follows. The lever was connected to the quick release mechanism, and  $17$  grammes of the initial load removed. Released without stimulus the muscle gave  $0.9$  gramme-cm. of heat: released at the moment a shock was given it gave  $66.9$  gramme-cm. The difference,  $66$  gramme-cm., is plotted as the full circle to the left of the heat curve. The completed curves show a striking parallelism between work and heat.

Another experiment of this kind was performed on the same muscle with  $33$  grammes initial load. Five negative after-loads were employed as shown in fig. 12. The initial load was so great that an appreciable but not a large

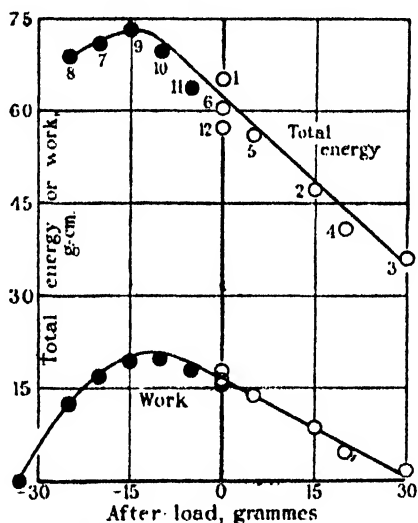


FIG. 12.—Sartorius of *R. esc.* 4.7.30. Same muscle as figs. 8 and 11. Single shocks at  $0^{\circ}$  C. Various positive and negative after-loads. Initial load  $33$  grammes. Full circles represent negative after-loads, hollow circles positive after-loads. Observations made as numbered. No corrections to work or heat for elastic potential energy of stretched muscle. For further details see text.

fraction of the work (see fig. 8) may have been done by the elastic extension of the muscle. Observations were made in the order of the numbers: the muscle was becoming gradually less excitable, and a mean curve is given. The striking thing about this diagram is the way in which, with negative after-loads, both work and heat continue to rise together and reach a maximum in about the same position. The negative after-load was applied by connecting the lever to the quick-release mechanism, removing the required weight, and releasing  $0.048$  second after the shock. An unstimulated release to  $-20$

grammes after-load gave 1·6 gramme-cm. of heat, but no correction has been made for this in the numbers plotted. The work in this unstimulated release was 1·7 gramme-cm.

It is clear from figs. 11 and 12 that in the region of initial loads where isotonic heat is considerably greater than isometric, work done (as Fenn claimed) largely determines the amount of energy liberated, even in the case of single twitches. There is not an exact parallelism: in figs. 11 and 12 the work in the region of negative after-loads comes back to the isometric level, but the heat does not; this, however, may be due to the fact that the external work recorded is not the whole work performed by the muscle—undoubtedly an appreciable amount has to be done in overcoming internal friction ("viscosity"), especially in rapid unloaded contractions. The existence, however, of a relation between work and heat in this region is just as clear as its absence in the other.

*The Effect of Releasing during a Twitch.*—If a muscle under a given initial load be stimulated isometrically and then released at some subsequent moment to carry out an isotonic contraction under the same load, the heat set free will in general be intermediate in amount between that of the isometric and that of the free isotonic contraction. The effect of the release depends on whether the load be small or great.

*Small Loads.*—In fig. 13, A, a semi-membranosus muscle at 21° C. under 5 grammes initial load was released at various moments after the shock and its heat-production measured. In this case the free isotonic heat was only 58½ per cent. of the isometric heat, and delaying the shortening caused an increase in the energy set free. This is the clearest proof that the energy set free in a twitch does not depend on the initial conditions alone, and if we assume that no negative heat is liberated at any stage in the contraction it allows us to make an interesting calculation of the minimum amount of heat liberated in an isometric twitch after any given moment. Release at 0·03 second, for example (fig. 13, A, at 21° C.), causes a diminution of 29 per cent. in the heat: after this moment, even if no heat at all were liberated in the isotonic twitch, there must still be 29 per cent. of the whole liberated in the isometric twitch. Release at 0·06 second causes a diminution of 12 per cent.; hence at least 12 per cent. of the heat in the isometric twitch is set free after 0·06 second. As the release is delayed the height of contraction decreases and less work is done. In spite, however, of the smaller amount of work the total energy set free is greater, a clear indication that in the region of small initial loads the heat production is not determined by the work. The same effect is shown in the lower curve

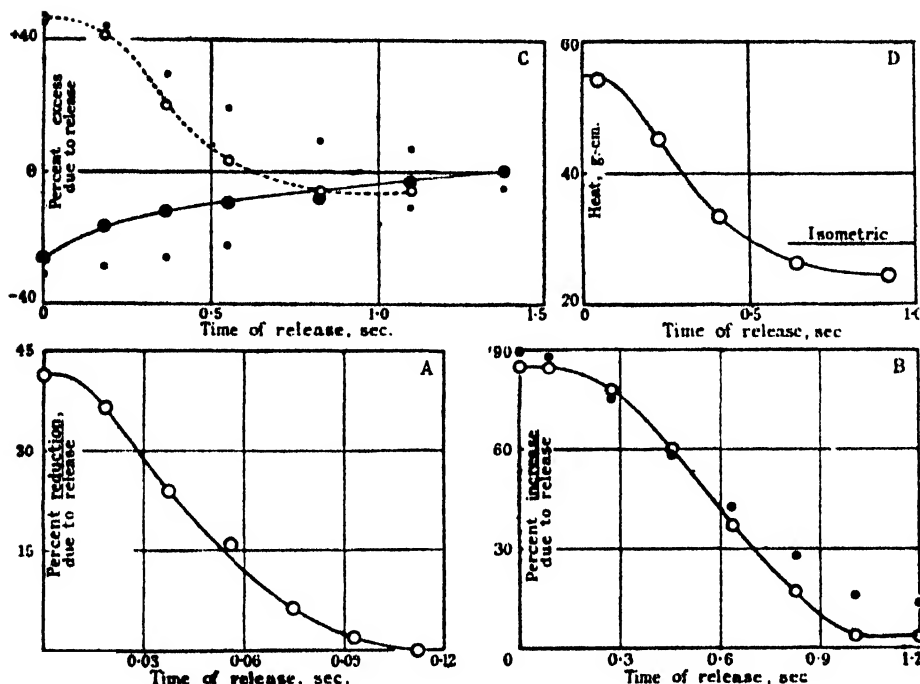


FIG. 13.—Quick release from isometric to isotonic contraction: single shocks. A, Semi-membranosus, 21° C., 5 grammes initial load. Heat *increased* by delay in release. B, Same muscle, later at 0° C., 20 grammes initial load (see also fig. 3). Heat *decreased* by delay in release. Full circles represent height of contraction. C, Sartorius, 0° C. Upper curve (broken line, hollow circles), 63 grammes initial load, heat *decreased* by delay in release. Lower curve (full line, full circles), 3 grammes initial load. Heat *increased* by delay in release. Small circles, contraction heights, arbitrary units. D, Sartorius, 0° C. Same muscle as figs. 8. 11 and 12. Released from 63 grammes (41.5 mm.) to 13 grammes (38.6 mm.). Net heat, i.e., after subtracting 7.5 gramme-cm., the heat of unstimulated release: isometric heat, as observed, without release. For details see text.

(full line) of fig. 13, C (sartorius, 0° C., 3 grammes initial load), where in addition the heights of contraction are recorded, diminishing with later release.

**Great Loads.**—In fig. 13, B, the same semi-membranosus as in fig. 13, A, but now at 0° C. and under 20 grammes initial load, was released at various moments. In this case (see also fig. 3) the free isotonic heat was 85 per cent. greater than the isometric, and delaying the shortening caused a decrease in the energy set free. This again shows clearly how the energy in a twitch may depend upon conditions existing long after the stimulus is over. In the same way and with the same assumption as above we can calculate that in this particular case at 0° C. at least  $76/1.76 = 43$  per cent. of the whole energy of

the isotonic twitch was liberated after 0.3 second, at least  $41/1.41 = 29$  per cent. after 0.6 second. The heights of contraction also are recorded, the extra heat runs very closely parallel to the work done (the load being constant). The same effect is shown in the upper curve (full line) of fig. 13, C (sartorius, 0° C., 63 grammes initial load), where again the heights of contraction are recorded.

This curve (as also fig. 13, D) illustrates a phenomenon which has been several times noticed, namely that late release may cause a deviation from isometric of the opposite sign from that given by earlier release. Whether the effect of early release be to make the heat greater or less, the curve may cross the base-line and return to it from the opposite side. A similar effect has also been described by Fenn (1924, p. 390), for the case of short tetanic contractions, and Azuma (1924, p. 342) made analogous observations. Their cause is not apparent, but they seem to occur in single twitches as well as in tetanic contractions.

The curve in fig. 13, D, deserves special mention in this connection. A pair of sartorius muscles at 0° C. (see fig. 8) was loaded with 63 grammes (41.5 mm. length) and released to 13 grammes (38.6 mm.). The unstimulated release gave 7.5 gramme-cm. of heat, due to the dissipation of elastic potential energy. This heat was subtracted from the heats observed (except the isometric). The following observations were made:—

Time of release: sec. after shock .....	0.046	0.23	0.41	0.64	0.92	Isomet.
Net heat: series →: g.cm. ....	54.4	44.7	33.3	26.4	24.7	29.4
Net heat: series ←: g.cm. ....	53.8	45.2	33.6	25.9	24.2	29.2
Mean heat: g.cm. ....	54.1	45.0	33.4	26.1	24.4	29.3
Mean contraction height: mm. ....	12.1	9.2	5.9	3.7	3.3	

Two series were made, with increasing and with decreasing intervals, and the means taken. The muscle was in very constant condition, and the agreement very good. The contraction released at 0.92 second was practically isometric—the shortening in an unstimulated release was 2.9 mm., nearly equal to the 3.3 mm. recorded—yet the heat was about 17 per cent. less than that of an isometric twitch.

### *Summary.*

1. The total energy set free in an isotonic twitch has been compared with that in an isometric one. The two are not equal except within a narrow range of initial loads. With small initial loads the isotonic heat may be much less than the isometric, with great initial loads much more. Differences between

different muscles are to be attributed to differences in their anatomical arrangements.

2. The conflicting results of several observers in this respect are due to variations in the range of initial loads within which they worked.

3. It is definitely untrue that the energy set free in the single twitch of a muscle depends only on conditions obtaining at the moment of stimulation. Release, for example, at a much later stage may considerably affect the energy, and—according to the initial load—the isotonic heat may vary almost from one-half to twice the isometric.

4. In a tetanic contraction the range of loads within which the isotonic heat is less than the isometric may close up so much as to be difficult to detect.

5. There are apparently two factors determining the energy liberated by a muscle even in a twitch. These are (*a*) the length of its fibres, not only at the moment of stimulation but throughout the contraction: and (*b*) work done. With smaller initial loads (*a*) is the more important, with greater initial loads (*b*) exerts the preponderant effect.

My thanks are due to Prof. Otto Meyerhof for calling my attention at an early stage to Dr. P. Rothschild's work, and to Dr. Rothschild himself for allowing me, some time before it was published, to see his manuscript.

During the course of this investigation Dr. Ernst Fischer, of Frankfurt, informed me in correspondence of recent experiments of his own on the oxygen consumption of muscles, from which he has reached the same conclusion as drawn here, viz., that the energy liberated in a twitch depends on two factors, the length of the fibres during the course of the contraction and the work done. His experiments will shortly be published in the "*American Journal of Physiology*."

#### REFERENCES.

- Azuma, R. (1924). 'Proc. Roy. Soc.,' B, vol. 96, p. 338.  
Fenn, W. O. (1923). 'J. Physiol.,' vol. 58, p. 175.  
Fenn, W. O. (1924). 'J. Physiol.,' vol. 58, p. 373.  
Gasser, H. S., and Hill, A. V. (1924). 'Proc. Roy. Soc.,' B, vol. 96, p. 398.  
Hartree, W., and Hill, A. V. (1928). 'Proc. Roy. Soc.,' B, vol. 104, p. 1.  
Hill, A. V. (1925). 'J. Physiol.,' vol. 60, p. 237.  
Meyerhof, O. (1930). "Die chemischen Vorgänge im Muskel." Springer. Berlin.  
Parkinson, J. L. (1926). 'J. Sci. Instr.,' vol. 3, p. 303.  
Rothschild, P. (1930). 'Biochem. Z.,' vol. 222, p. 21.

*Observations on the Living Eggs of the Rabbit.*

By GREGORY PINCUS.\*

(Communicated by F. H. A. Marshall, F.R.S.—Received May 14, 1930.)

(From the Institute of Animal Nutrition, School of Agriculture, Cambridge University, and the Strangeways Research Laboratory, Cambridge.)

[PLATES 10-13.]

In the course of certain observations on the fertilisation of rabbit ova *in vitro* it soon became evident that, contrary to normal expectation, unfertilised ova will undergo varying degrees of development, much of the development being indistinguishable morphologically from the development of fertilised ova *in vivo*. It was decided, therefore, to undertake a comprehensive study of the history of tubal ova and of their behaviour *in vitro*. Advantage was taken of the fact that the rabbit ovulates regularly at about 10 hours after copulation (*e.g.*, Heape, 1905; Hammond and Marshall, 1925; Walton and Hammond, 1928) to obtain ova of known age. Unfertilised ova were obtained by mating non-pregnant rabbit does to vasectomised bucks, and the eggs liberated from the ovary were recovered from the tubes by washing them out with sterile isotonic Pannett-Compton solution (Pannett and Compton, 1924). By this method eggs were obtained from the tubes from 11 to 72 hours after copulation. Between 72 and 96 hours after copulation the unfertilised ova enter the uterus and similar uterine washings were made to recover such eggs. Each doe used was mated to two or three vasectomised bucks. That these animals had been successfully operated upon and were incapable of ejaculating spermatozoa was already shown by the fact that no spermatozoa were found in smears taken from the vulva of the doe immediately after copulation and eggs obtained from these matings never showed the characteristic features, to be described later, of normally fertilised eggs. Some, moreover, showed no evidence of development *in vivo*. Ova fertilised *in vivo* and used for control experiments were similarly obtained, but from matings made to normal fertile bucks. To obtain the uterus and tubes the doe was killed and these organs removed with the ordinary precautions for attaining sterile conditions.

\* National Research Council Fellow.

In the few experiments in which ovarian ova were employed the eggs were obtained by pricking ripe follicles with a sterile needle and floating the emerging eggs into sterile Pannett-Compton solution.

Except where otherwise noted ordinary tissue culture methods were employed for *in vitro* growth. The usual precautions were taken to ensure sterile culture conditions. The medium ordinarily employed consisted of a clot formed by mixing equal parts of rabbit blood plasma and 7 to 8-day chick embryo extract. In experiments I to XII chicken blood plasma and chick embryo extract were used. For certain purposes, as hereinafter noted, the medium was varied, but the combination of rabbit plasma and chick embryo extract proved the most advantageous. The culture clot was formed in the bottom of a watch glass placed in a moist chamber. The moist chamber was made by soaking sterile cotton wool placed in a sterile petri dish with sterile water. For purposes of microscopical observation special petri dish covers were made in which a round hole about an inch in diameter was cut. Sterile coverslips were placed over this opening and sealed on to the petri dish with paraffin wax. A hole was cut in the cotton wool directly under the watch glass to allow unobstructed illumination with a bright light when needed.

The problem naturally divides itself into these aspects: (1) the history of unfertilised ova *in vivo*; (2) the history of fertilised ova *in vivo*; (3) the behaviour of fertilised ova *in vitro*; (4) the behaviour of unfertilised ova *in vitro*; (5) the fertilising capacity of rabbit ova.

### *The History of Unfertilised Ova in vivo.*

Eggs were recovered from the Fallopian tubes as described above. To obtain ova at from 11 to 16 hours after copulation it is advisable to wash from the uterine ends of the tubes towards the fimbriated ends. In order to do this a portion of the uterus is cut off with the tubes and the pipette containing the washing fluid is inserted into the uterine lumen. To obtain eggs from 17 hours after copulation and onwards the washing may be done from the fimbriated ends. It is advisable to use a rather fine pipette when washing from the uterine end and a fairly broad one when washing from the fimbriated end. Care must be taken to cut the mesenteric connections of the tubes to prevent buckling during washing, and so allow of a free flow of fluid through the tubes.

On ovulation the eggs emerge from the follicles surrounded by the discus proligerus. From one to eight eggs are liberated by a single ovary. In my



experience ovulation occurs invariably in both ovaries though one ovary may produce but one egg and the other many more. The highest number ovulated was 13, 7 from one ovary and 6 from the other; the lowest number was 3. An average of eight eggs was recovered from the does used in these experiments.

The freshly ovulated ova enter the tubes and become massed together, due to the adherence of the sticky masses of cumulus cells. By 11 hours after copulation (about 1 hour after ovulation) this mass of cumulus cells containing the ova becomes securely lodged in the narrower portion of the tubes just below the broad, fimbriated end. On washing from the uterine end of the tubes this mass (see fig. 1, Plate 10) is first ejected, then the washing fluid. The ova remain thus massed together until about 17 hours after copulation, an occasional ovum separating out of the mass as early as 16 hours after copulation. Fig. 2 is the photograph of an ovum still imbedded in the mass of follicle cells at 16 hours after copulation. Fig. 3 is the photograph of the single one of the 10 ova removed at the same time as that of fig. 2 that had separated out of the mass. Note a number of follicle cells still clinging to the egg. As they separate out of the cumulus mass the eggs emerge surrounded more or less by a few adherent follicle cells, and proceed down the tubes where these few adherent cells are lost. At 20 hours after copulation all the adherent cells are gone and a thin layer of albumen is laid down about the zona pellucida. Eggs washed out at this time show very clearly the transparent, shining zona pellucida about the yolky, granular egg cytoplasm, with an extremely thin albumen layer surrounding the zona (see fig. 4). The process involving the separation of the eggs out of the cumulus mass and the clearing off of adherent cells thus involves a period of about 3 hours. When eggs are washed out during this period one observes in a single washing all the stages described, eggs completely clear of adherent cells being preponderant toward the end of the period. One may even find an occasional egg still surrounded by adherent cells as late as 20 hours after copulation. Long (1912) in describing the methods for recovering rat and mouse ova obtained ova in the cumulus mass as late as 25 hours after parturition. In this form ovulation commonly occurs at about 14 hours after parturition. This time of ovulation is not, however, invariable so that the ova described may not have been in the cumulus mass for 11 hours as one would deduce. They certainly do remain in this mass for 6 to 8 hours at any rate which is about the same period of time observed in the rabbit.

It is important for reasons that will be obvious later, to note that by 20 hours after copulation all rabbit ova are free of follicle cells and have begun

to accumulate a layer of albumen. By 24 hours after copulation this albumen layer is quite appreciable (see fig. 5). Subsequently the ova descend to the uterine end of the tubes acquiring in their passage successive layers of albumen so that the albumen layer may eventually become several times the thickness of the egg itself (see figs. 7, 8 and 9). The zona pellucida no longer presents the clear, shining appearance observed before the deposition of albumen. Most of the ova recovered from the tubes contain at least one polar body, occasionally two or even three. In some cases I have been unable to observe any, but am inclined to ascribe this to faulty observation as the eggs often come to rest with the polar body hidden.

The eggs enter the uterus between 72 and 96 hours after copulation. No more albumen is added and the eggs undergo rapid disintegration. I have, in fact, found it very difficult to recover unfertilised ova from the uterus. In no case have I been able to obtain the full complement as indicated by the corpora lutea count. They are either rapidly resorbed or washed out into the vagina. The cytoplasm of eggs recovered from the uterus shows distinct evidences of degeneration (figs. 8 and 9).

On three occasions the writer has observed unfertilised rabbit ova which gave evidence of a certain degree of development *in vivo*. The first occasion occurred at the Bussey Institution of Harvard University where Dr. P. W. Gregory was kind enough to show me in, I believe, the spring of 1928, a set of unfertilised ova recovered from a rabbit doe belonging to the stock of Prof. W. E. Castle. All the ova in this set had apparently proliferated a number of small cells comparable to polar bodies. I had an opportunity to make a count of the chromosome complement of one of these ova and found 22 chromosomes, apparently diads, fairly widely scattered in the centre of the main body of the egg. Unfortunately I have no record of the age of these ova, but I have twice in my own series made similar observations. The egg of fig. 10 was taken from a rabbit doe 41 hours and 30 minutes after sterile copulation, and resembles almost exactly the ova shown me by Dr. Gregory. Six of the eight ova from the same batch showed somewhat similar development, one or two exhibiting rather irregular cell outlines in the body of the egg. It is notable that these ova were recovered from a doe mated in Cambridge and transported to London by train before she was killed. In the third instance I observed a single ovum showing many small polar-body like cells out of nine recovered at about 36 hours after copulation. This doe, too, was mated in Cambridge and transported to London before killing. In all the other unfertilised eggs, amounting to several hundreds, which I have observed no *in*

*vivo* development occurred, and in no instance was the development comparable to the regular segmentation of fertilised ova.\*

*The History of Fertilised Ova in vivo.*

At 11 hours after copulation eggs obtained from does mated to fertile bucks are also found in the cumulus mass. This is especially true of large does where ovulation apparently occurs slightly later than in smaller does. In the case of one small doe which was killed at 11 hours after copulation the eggs were not in the cumulus mass but separate from each other though still heavily surrounded by the follicle cells. But the masses washed out at 11 hours after fertile copulation present a different appearance from those obtained from the tubes of does mated to vasectomised bucks. The masses in the latter case are fairly smooth in appearance, whereas in the former case the edges of the mass are quite ragged, and occasionally one observes sperm amid the follicle cells. By 11½ hours after copulation most of the eggs are separated out of the cumulus mass, but they are still heavily surrounded by cumulus cells. By 12 hours and 20 minutes to 13 hours after copulation (see fig. 11) most of the adherent cells are reduced to a layer one or two cells in thickness although occasionally one finds one or two ova still in the cumulus mass. By 14 hours after copulation all the eggs are practically free of adherent cells, and at 15 hours after copulation they are completely free, and an extremely thin albumen layer is formed about the egg. At this time the ova resemble exactly those unfertilised ova recovered from the tubes at 19 to 20 hours after sterile copulation (see fig. 4) except that one may observe from 12 to 100 sperm stuck in the zona pellucida. Practically all of these sperm lie in the zona with their heads at an angle to the axis of the egg rather than perpendicular to it. Such sperm may be detected as early as 11½ hours after copulation in those eggs most clear of adherent follicle cells, but they are most numerous in eggs removed at 14 to 15 hours after copulation. It is thus obvious that the fertilising spermatozoon must enter the egg somewhere between 11½ and 13 hours after copulation. Since ovulation occurs at about 10 hours after copulation it takes from 1½ to 3 hours for the fertilising spermatozoon to enter the egg. It is evident from these findings that much of this delay is due to the necessity for removing and penetrating the surrounding cumulus mass. A further consideration of this point is given in later sections.

\* Charlton (1917) has described the fragmentation of certain tubal ova of the white mouse into morula-like bodies after the ova had been in the tubes about two days. See also Long and Evans (1922) on the rat.

Eggs recovered from the tubes from 15 to 20 hours after copulation are all in the one cell stage. A second polar body is put off by about 17 to 18½ hours after copulation (see fig. 12). From 18½ to 20 hours after copulation one observes a large clear area in the centre of the egg which divides into two (evidently the cleavage nuclei). By 21 hours after copulation some of the eggs have begun to segment, and all of them are in two cells by 24 hours after copulation. The subsequent history of fertilised ova is too well known to be gone into in detail (Assheton, 1894 ; Lewis and Gregory, 1929). I might add, however, that my findings confirm those of previous writers as to time of subsequent segmentations (see Appendix).

*The Behaviour of Fertilised Ova in vitro.*

This section is devoted to ova taken from does which had been mated to fertile bucks. A consideration of these ova is advisedly given precedence over unfertilised ova because it is important, in considering the behaviour of unfertilised ova *in vitro*, to keep in mind that fertilised ova almost invariably segment with great regularity in culture. Most of the experiments described in this section were, in fact, conducted as controls to the experiments with unfertilised ova, the eggs, with exceptions to be noted, being placed in culture at the same time and under the self-same conditions.

The complete data are given in Appendix (Table I). It will be noted that one-celled ova recovered from the tubes from 14 hours after copulation to 21 hours and 20 minutes after copulation almost invariably segment regularly in culture. The one notable exception is shown in the data of column 10. These ova were used chiefly for determining the effect of variations in technique which proved to be unsuitable. Even in these ova I was able to observe the formation of two cleavage nuclei, but no segmentation. If then we exclude the data for column 10 we arrive at the results for one-celled ova, shown in Table I below.

Table I. - Segmentation of Fertilised Ova *in vitro*.

Age of ova (hours after copulation).	Number divided.	Number undivided.	Percentage divided.
11 to 12	4	19	17.4
12 to 13	13	6	68.4
14 to 16	35	2	94.6
17 to 22	21	0	100.0

Now the significance of these data will be realised if we keep in mind the morphological findings given in the preceding sections. Examination of the conditions of the surrounding follicle cells and the zona pellucida led to the conclusion that the fertilising spermatozoon entered the egg somewhere between  $11\frac{1}{2}$  and 13 hours after copulation. Now if the subsequent processes of fertilisation and segmentation proceed normally in culture then all ova recovered after the critical period of 13 hours after copulation should segment *in vitro*. The data of Table I show that this is substantially the case. They show, furthermore, that practically no fertilisation takes place between 11 and 12 hours after copulation. The data of experiment XXIX (Appendix, Table I, No. 2) are particularly significant in this connection because the ova were placed not in the regular culture medium but in a Ringer's solution buffered to  $p_H 7.14$ . In a series of controls carried on at the same time none of the ova (unfertilised) segmented in the Ringer's solution. It follows, therefore, that since unfertilised ova will not segment in Ringer's solution only the ova recovered at 12 hours and 20 minutes after copulation were fertilised and only six out of these eight. The four ova of the 11 to 12 hour group that did segment were grown in the regular culture medium, in which, as shall be shown later, a certain percentage of unfertilised ova normally segment any way; and furthermore, of these four ova two were recovered at 11 hours and 55 minutes after copulation.

It is thus obvious that a period of 2 to 3 hours is normally required for the spermatozoa to penetrate through the surrounding follicle cells and enter the egg. This is a consideration of some importance for the subsequent section dealing with the fertilising capacity of the rabbit ovum.

It is, of course, regrettable that sufficient time was not available for complementary cytological studies. It is hoped that such studies will be made in the near future, but it should be stressed that these experiments are considered primarily as controls to the experiments with unfertilised ova detailed below.

No attempt has been made in any of these experiments to prolong the life of the ova in culture. Examinations of the cultures were generally made at 24-hour intervals over a period of 2 days. The examinations were ordinarily carried on under the microscope at room temperature, and in a number of instances the cultures were returned to the incubator (heated to  $37\frac{1}{2}^{\circ}$  C.) after 15 to 50 minutes of observation. It is obvious that this exposure to room temperature cannot have been beneficial to the cultures. It became evident, nevertheless, that segmentation does not proceed perceptibly after the ova have been in culture for 36 hours although this varies somewhat with the age

of the eggs employed. Lewis and Gregory (1929, *a*) record the development of one-celled rabbit eggs to the eight-cell stage in 18 to 19 hours of culturing. They also have observed development of the inner cell mass (1929, *b*) in 4 days. It will be noted that some of the ova here recorded developed as far as the morula. The variation is, however, great, and it is not deemed feasible to consider the question of viability on the basis of these experiments.

Data on the nature and rates of division of fertilised ova are now being accumulated in a series of cinematographic records which will be recorded elsewhere. The actual process of segmentation, however, takes about 10 minutes for its completion, varying between 9 and 15½ minutes (see figs. 13-17). The time at which the first segmentation of fertilised ova occurs varies with the age of the ova, those recovered soon after fertilisation segmenting later than those recovered some time after fertilisation.

#### *The Behaviour of Unfertilised Ova in vitro.*

The data for these experiments have been divided into two tables in the Appendix (Tables II and III). It is the data of Table II that will be discussed in this section, those of Table III being reserved for presentation in the succeeding section.

It may be noted in the first place that development proceeds in a number of media. While no critical experimentation was undertaken, the combination of rabbit blood plasma and chicken embryo extract was found to be technically the most satisfactory. I do not doubt that improvements can be devised, but the technique used was found adequate for an investigation upon which certain necessary limitations were placed.

The primary and surprising fact evident from the data is that a majority of the ova placed in culture underwent a certain degree of development, so that out of 213 eggs cultured, 136 or 63·8 per cent. are classified as having "divided," the term "divided" including any degree of observable development beyond the one-celled state of the ova as recovered from the animals. It was the primary objective of the investigations described in this section to ascertain the nature of the various degrees of development undergone *in vitro* and to establish any relationship that might exist between the age of the ova and the nature of the development. Before undertaking any detailed analysis of the data at hand it is deemed advisable to describe the various types of development observed. For this purpose I have assembled a representative series of photographs (figs. 18 to 32). It was unfortunately impossible to photograph the material in the incubator, and the removal of the cultures to the

microscope used for photographing resulted in certain changes in the ova, consisting chiefly in a change in the viscosity of the egg protoplasm and a slight shrinkage. Furthermore, the exigencies of photographing the varied material at hand required the exercise of a certain amount of ingenuity in the actual technique of photography so that from a photographic point of view the results are by no means ideal. Otherwise the photographs are faithful representations of the ova as cultured. The ova as represented in these photographs are magnified 130 to 150 diameters. The few ova that I have measured varied from 119 to 132 microns in diameter, excluding the albumen coating.

The ova observed in the two-cell stage varied in appearance as shown in figs. 18 to 20. The great majority of them resembled that of fig. 18, and showed usually one, sometimes two or three, polar bodies. The ovum of fig. 20 was photographed after the egg had been in culture 22 hours. It was subsequently replaced, and when examined 24 hours later had formed eight cells quite regular in appearance. Note is made of this fact because it indicates that ova segmenting irregularly at the first division may eventually assume an appearance characteristic of ova undergoing quite regular division. The ovum of fig. 21 was photographed just as segmentation from two to three cells was being completed. One of the two blastomeres had not quite rounded out at the time of photographing. The segmented ovum of fig. 22 is also in three cells. When first examined after 23 hours of culturing no segmentation had occurred; 5 hours later the ovum had divided as photographed. The ova of fig. 22 were recovered at 18 hours and 15 minutes after copulation and were still surrounded by a number of follicle cells. They were placed *vis-à-vis* in culture and the outgrowing follicle cells of each ovum became intermingled and caused the compression of the ova seen in the photograph.\* Fig. 23 is a photograph of a typical four-celled stage, exactly comparable to the four-celled stage of fertilised ova (see fig. 14). The number of polar bodies in such ova vary from one to three. Again, the great majority of ova observed in four cells presented the regular appearance of the ovum of fig. 23. Fig. 24 represents an ovum containing seven cells in which one of the four blastomeres of the four-celled stage divided twice while the others remained quiescent. Such differential division may begin after the two-celled stage as illustrated by fig. 25, in which one of the original two cells has remained quiescent while

\* The outgrowth of follicle cells in culture has been observed very many times. The cells above and below the ova creep out on to the culture clot in spike-like formations of spindle shaped cells much like fibroblasts. In addition typical wandering cells have been observed.

the other divided in two, and one of the two cells formed divided twice to form four small cells. There is also photographed the single polar body of this ovum. Fig. 26 represents another case in which one of the early blastomeres has remained quiescent while the others have gone on dividing at a rapid rate. Some such process is responsible for most of the irregular segmentations observed. At the same time segmentation may proceed in a manner comparable to that of normal fertilised ova *in vivo*, so that one may observe in the same culture the different types described. Fig. 27 is a photograph of an ovum segmented to about 20 cells and apparently with a marked degree of regularity. When we come to consider ova segmented into 20 and more cells the interpretation of the course of their development becomes difficult because of a peculiar complication. The ovum of fig. 28 offers a pertinent illustration. It was recovered from the tubes at 37 hours after copulation and was in the one cell stage. Six hours later it presented the appearance shown in the photograph. It has apparently segmented into about 36 cells in the course of 6 hours. This means astonishingly rapid segmentation. As a matter of fact what probably occurred was a complex fragmentation of the entire ovum. In the course of filming an ovum recovered at 29 hours and 20 minutes after copulation I had the opportunity of observing such fragmentation. After an initial period of quiescence the ovum underwent a long period of activity which resulted in an appearance suggesting the presence of many small blastomeres. The "cells" of this fragmented ovum, however, were not at all distinct in form or outline. I have from time to time observed "many-celled" ova in culture that presented this vagueness of cell outline, but have also observed well advanced ova in which the component blastomeres were as distinct and clear as in the normal fertilised ovum. Interpretation must, therefore, proceed slowly until the exact mechanics of division *in vitro* is thoroughly investigated. A certain amount of light, however, is shed on the problem by the consideration given below to the relation between the age of the ova and the nature of the development observed. Figs. 29 and 30 are photographs of two ova recovered at 73 hours and 40 minutes after copulation. They were photographed after having been 45 hours in the same culture. Note the remarkable regularity of the cells of the ovum of fig. 30. The ova of figs. 31 and 32 represent types ordinarily described as "with many polar bodies." Both have a very large single cell, beside which lie a number of very small "cells" comparable in appearance to polar bodies. Very often this group of "polar bodies" resembles an irregular indented cytoplasmic mass, and I have actually seen it formed as such a mass budded or divided



off from the main body of the cell. This represents the extreme of irregularity observed.

The foregoing account has been given irrespective of the age of the ova figured. It remains for us to ascertain if any relation does exist between the age of the ova cultured and the nature of their development. Before proceeding to a detailed inquiry, however, it must be pointed out that the various types of ova described and figured in the photographs have been observed in ova of all ages so that no absolute correlation exists. I have, however, compiled from my data and the available notes the following table. Ova have been considered as segmenting regularly only when the cells of the two, four, eight, and sixteen cell stages have been of equal size, or when one could obviously trace the regular descent of the cells in ova exhibiting intermediate stages. In the case of ova exhibiting many cells only those showing clear cell outlines and cells of equal size have been classified as "regular."

Table II.—Effect of Age of Ova when removed from Doe on subsequent regularity of division *in vitro*.

Age of ova (hours after copulation).	Regular.	Irregular.	Percentage regular.
(1) 11 to 17	26	8	76.4
(2) 17 to 21	37	16	69.8
(3) 24 to 96	10 (?)	26	27.7
All ova	73	50	59.3

In Table II I have collected the data into three groups as follows: (1) Ova recovered when practically all were in the cumulus mass; (2) ova separating out of cumulus mass and not yet covered with albumen; (3) ova covered with the albumen deposit. It is obvious from these data that the percentage of ova segmenting with any semblance of regularity decreases perceptibly with the age of the ova. In the group of ova recovered at 24 to 96 hours after copulation 16 of the ova classed as irregular exhibited one large cell and "many polar bodies." In fact, 23 or about half of all the ova called "irregular" are of this type. A number of ova, particularly in the 24 to 96 hour group exhibited "many polar bodies" and a varying number of larger cells. The rest of the ova classified as irregular were either "many-celled" with indistinct cell outlines, or contained cells of unequal size traceable, probably, to the differential division of early blastomeres.

Now this fact that the younger ova tend to segment regularly is, I believe,

related to the state of the egg cytoplasm. The older ova undoubtedly undergo a certain degree of degeneration as they progress down the tubes, and the degree of cytoplasmic degeneration is probably related to the regularity of the subsequent development in culture. The problem is unfortunately complicated by the fact that all ova in culture stop segmenting and degenerate after some time. My observations lead me to believe that practically no development occurs after the ova have been in culture for 36 hours. The time in which the ova may exhibit their potentialities for parthenogenetic development is, under the conditions of these experiments, therefore extremely limited. The surprising fact is that such a large proportion of the ova do exhibit a degree of development that must be classified as parthenogenetic.

The morphology and cytology of parthenogenetic ova have been studied in a number of invertebrate forms where parthenogenetic development has been induced by various methods of treatment. In almost all cases a very large proportion of the parthenogenetic ova exhibit marked irregularities in development (*e.g.*, Wilson, 1901; Scott, 1906; Morris, 1917). In fact all the irregular types described here have been observed in artificially parthenogenetic invertebrate ova. The proportion of regular divisions observed in these ova compares favourably with those observed in invertebrate ova, with the possible exception of the sea-urchin eggs, a very large proportion of which (as much as 100 per cent.) may develop regularly into swimming larvæ (Hindle, 1910; Loeb, 1913).

It has not been possible to make any extensive cytological study of the ova described here. The few sectioned and stained eggs obtained, indicate that in ova segmenting regularly the nuclei and cytoplasm are normal in appearance (see fig. 33). In ova segmenting irregularly the situation is apparently rather complicated. There are obvious evidences of degeneration. Some cells contain nuclei, others do not, and the cytoplasm is often quite degenerate. I have also observed ova with several nuclei and no distinct cell divisions. In the case of one fairly regular ovum I was able to count the chromosomes of an incomplete metaphase plate. There were at least 37 chromosomes comparable in morphology to normal somatic chromosomes. This would indicate that the chromosome number was probably diploid (see Painter, 1926), and such a condition may have arisen by the fusion of two early nuclei (*e.g.*, the pronucleus with a polar body nucleus as described by Morris, 1917). I do not wish to stress these cytological findings, however, for two reasons: first, they are few and obviously incomplete; second, a number of the ova were fixed when the ordinary *in vitro* degeneration may have begun. The cytological

aspects of the problem undoubtedly require exhaustive investigation involving as they do the relation of maturation to parthenogenesis, the nuclear-cytoplasmic relations of parthenogenetic ova and so on. It is sufficient here to indicate that a number of the segmentations recorded involved the regular participation of the nucleus in a manner comparable to the normal.

Several recent reviews (Kampmeier, 1929; Branca, 1925; and Hartman, 1926) happily absolve the writer from any extended discussion of the much-laboured question of mammalian parthenogenesis. With the exception of a single observation reported by Champy (1927) no record of experimental parthenogenesis has been found. The commonest observations consist of the record of segmented or fragmented ova found in the ovaries of various mammals. The occurrence of ova which cannot strictly be called one-celled is more frequent apparently in the ovaries of some forms than in others. The segmentation of the various ova of the water vole, as observed by Sansom (1920), is an exceptionally clear case. Such phenomena are exceedingly rare in the rabbit. The observations of Asami (1920) on the atretic follicles of the rabbit ovary give no indication of the occurrence of segmentation. Mr. John Hammond in a personal communication declares that in his examination of several hundred sectioned and stained rabbit ovaries he has never observed a single case of intra-ovarian segmentation (see Hammond and Marshall, 1925). When one contrasts this situation with the results obtained from the *in vitro* development of rabbit ova it is obvious that in the growing of rabbit ova in culture a stimulus to development is obtained that does not ordinarily exist *in vivo*. With the exception of the three isolated instances cited above no record has been discovered of the development of unfertilised tubal ova *in vivo*.

One question is common to the subject of ovarian parthenogenesis and the study here described, and that is—are we observing true parthenogenesis or merely degenerative fragmentation? It must be admitted at once that certain of the results described here are unquestionably attributable to degenerative fragmentation. This is particularly true of the older tubal and uterine ova. Such a conclusion is supported by the increased frequency of anomalous types of division associated with the increasing age of the ova. But the writer holds that the many cases of regular cleavage observed are truly parthenogenetic. One may ask why does growing these ova in culture lead in certain cases to degenerative fragmentation and in others to true parthenogenesis? It may be pointed out, first of all, that the line of demarcation between the two is by no means distinct. The participation of the nucleus in certain of the cases of anomalous division introduces a factor for which the mere label “de-

generation " does not suffice. It may be argued that the term parthenogenesis should be applied to all cases wherein nuclear division occurs in unfertilised ova. But we are not concerned here with matters of definition. The clue to the problem lies, we believe, in the very frequent occurrence of regular cleavage in the younger ova. In such ova we are dealing with a cytoplasm that must be as " healthy " as the cytoplasm of fertilised ova of the same age. In the older tubal ova a certain amount of cytoplasmic degeneration has probably taken place. It is suggested, therefore, that the regularity of cleavage is correlated with the degree of cytoplasmic degeneration. In those younger ova which show anomalous divisions a certain amount of degeneration must have taken place either *in vivo* or in culture. Conversely, in the few older ova showing regular segmentation cytoplasmic degeneration was at a minimum. Such conclusions, however, must be taken as purely inferential. The necessity for further investigation is evident.

Considerations of parthenogenesis or fragmentation do not, however, explain the discrepancy between *in vivo* and *in vitro* behaviour. Why should any development of unfertilised ova be so rare *in vivo* and so extremely frequent *in vitro* ? There are a number of factors concerned in the transference of ova from the tubes to the culture. Almost all of these may, we believe, be ruled out of consideration as causative agents.

First, there is the fluid used to wash out the eggs. Is there some effect of the balanced salt solution ? I believe not because (1) development of unfertilised ova does not go on, as far as I have been able to discover, in balanced salt solution alone, and (2) eggs washed out with serum or defibrinated plasma do develop *in vitro*.

Secondly, the eggs are ordinarily washed out of the tubes at room temperature. Is it then the sudden lowering of the temperature that is responsible for the subsequent development ? Sudden temperature changes have resulted in the parthenogenetic development of invertebrate ova, chiefly brief exposures to high temperatures. But I have in a few instances washed eggs out with warm fluid into warm vessels, and transferred them immediately into warm cultures in the incubator. These eggs have segmented *in vitro*. It is possible, however, that in the handling their temperature fell below body temperature despite the precautions taken. So the temporary change of temperature is not entirely ruled out as a causative agent, though rendered improbable.

Thirdly, is the culture medium itself the furnisher of the stimulus to development ? In a few experiments conducted with ova placed in balanced salt solution alone no development was observed. Nevertheless ova will develop

in embryo extract alone or in blood plasma alone. Therefore neither of the ingredients of the culture medium is responsible in itself for the development. I am inclined to believe that the culture medium acts as a nourishing rather than a stimulating agent.

Fourthly, is a shock due to handling the stimulating factor? Eggs washed out while still embedded in the cumulus mass are never touched by any of the glassware used and the mass itself floats easily in the washing fluid, and yet eggs so embedded do develop *in vitro*.

Fifthly, is there some substance present in the Fallopian tubes and ovaries which inhibits development *in vivo*, and is this inhibitor not removed in the washing fluid? Or is it a substance continuously present in the tubes and ovaries and not present in the culture medium? But what is this substance that specifically inhibits the *in vivo* segmentation of ova while the somatic cells do undergo development?

There is one substance present in comparatively small amount in the tubes which is present in large amount in the culture medium. That substance is oxygen. The ova develop in cultures to which the air has free access, and into which it presumably diffuses freely. According to Campbell (1924) the oxygen tension of the abdominal cavity of the rabbit is about 40 mm. Hg as compared with 150 mm. Hg, which is the oxygen tension of the air. Furthermore, the  $\text{CO}_2$  tension of the abdominal cavity is about 50 mm. Hg as compared with a tension in the air of almost 0. The fluids of the abdominal cavity are undoubtedly continuously swept into the Fallopian tubes so that the oxygen tension of the tubes must be about the same as that of the abdominal cavity. Furthermore, such small pieces of tissue as rabbit ova undoubtedly permit of very ready oxygen and carbon dioxide diffusion. Just how such an increase in oxygen tension and decrease in carbon dioxide tension should act as a stimulus to division is, of course, problematical. In invertebrate eggs, increase of oxygen tension is not effective in producing parthenogenetic development, but it must be remembered that they live in an entirely different environment in this respect from rabbit ova.

If the change of oxygen and carbon dioxide tensions is the responsible factor for development it should follow that unfertilised ova placed in Ringer's solution should develop. It is highly probable, however, that such ova died before any development can be initiated. The observations of Long (1912) on mouse ova indicate that these ova begin to die after they have been in Ringer's solution for 12 hours. My observations indicate that the regular parthenogenetic division ordinarily begins to appear when the ova have been

in culture for 8 to 9 hours. Older ova which undergo a certain development before this time have not been cultured in Ringer's solution.

There is, finally, the possibility that parthenogenetic development may be induced by hypertonicity of the culture medium. The ova are cultured in a medium from which evaporation of water is possible even though it is placed in a moist chamber, as evaporation from the petri dish into the air undoubtedly occurs. Such evaporation and consequent parthenogenesis have occurred in the case of invertebrate ova (*cf.* Just, 1928). On the other hand, there is also the possibility of the diffusion of water into the culture medium from the water vapour of the moist chamber.

A note concerning the rate and nature of parthenogenetic segmentation is not amiss here. Final determinations await the results of a cinematographic record now in progress. It may be stated, however, that the first segmentation division of unfertilised ova dividing regularly into two cells occurs from 6 to 14 hours after their being cultured. The cytoplasm, which may on recovery contain a number of large pigmented yolk granules becomes ordinarily uniform and clear after 3 or 4 hours in culture, and this clearing up of the cytoplasm generally indicates that a segmentation is to follow. I have included prints of two 9-minute strips of film in figs. 16 and 17. Fig. 16 shows a fertilised ovum dividing *in vitro*; fig. 17 an unfertilised ovum. The time taken for segmentation is about the same in each case. Note that the division is into two unequal cells (*cf.* Lewis and Gregory, 1929, *a* and *b*). The cinematograph records demonstrate also considerable cytoplasmic movement preceding and during the segmentation but none of the pronounced bubbling observable in cells ordinarily dividing *in vitro*. It cannot be stated at present whether there is any pronounced difference between the cytoplasmic movements and rates of segmentation in fertilised and unfertilised ova.

Figs. 34 and 35 illustrate a phenomenon occasionally observed in these experiments. The ovum of fig. 34 was photographed after it had been removed from an incubator into the room and then placed in the incubator containing the cinematographic apparatus. It was evidently about to segment into three cells (it contains also "many polar bodies"), but a photograph taken 33 minutes later (see the clock) revealed that this segmentation had not taken place. Whether the temporary exposure to room temperature was responsible for the reversal I do not know although I have observed an exactly similar phenomenon under the same conditions.

*The Fertilisable Condition of Rabbit Ova.*

The chief data for this section are in Appendix, Table III, and include only ova taken from the does up to 21 hours after copulation. By 21 hours after copulation all ova are free of adherent follicle cells and are surrounded by a coating of albumen which the sperm cannot penetrate. I have many times attempted to cause fertilisation of ova with albumen coatings, but have in no instance observed even the slightest degree of entrance of sperm into the albumen.

The technique of insemination *in vitro* may be outlined as follows. Sperm are taken from the vas deferens of fertile rabbit bucks, the vas being removed under sterile conditions immediately after killing the buck. The ova are placed on the culture clot as previously indicated. A small drop of sperm is squeezed out of the vas deferens on to a sterile watch glass, and this sperm suspension is picked up by a very fine sterile capillary pipette. The sperm suspension is expelled from the pipette on to the culture clot in the immediate vicinity of the ova. The latter operation can be carried on under the dissecting microscope. If the suspension is a "good" one the sperm become immediately active on being placed in culture; they spread out in all directions from the original drop, and remain active for a long time. In cultures made up with rabbit plasma I have detected motile spermatozoa as late as 30 hours after their being placed there.

The ensuing events vary with the state of the ova, particularly as regards the adherent follicle cells. Where the latter are numerous the sperm cannot at first reach the ova, and they move about at the periphery gradually causing the detachment of the follicle cells (see Long, 1912). They are not always successful, however, in rendering the eggs completely bare, particularly if there is a very large mass of surrounding cells and a not very heavy sperm suspension. Furthermore, the follicle cells in the immediate vicinity of the ova may begin to grow out on to the culture in a fairly short time. When, however, a fairly heavy sperm suspension is used the follicle cells are sufficiently detached in the course of 1 to 3 hours to permit the sperm to reach the zona pellucida. It is very difficult to observe the actual penetration of a spermatozoon under the microscope, first, because the follicle cells obscure the ova, and, secondly, because the ova are almost continually in motion. There is a third phenomenon which may entirely prevent the entrance of spermatozoa. I have observed this chiefly in ova placed with sperm in a liquid medium, and it consists apparently, of the compacting of certain of the adherent cells im-

mediately about the ova. I have, however, on two occasions observed the penetration of the ovum by a spermatozoon. The spermatozoon in each case was found with its head partially in the zona and at right angles to the axis of the egg. The tail movements were fairly vigorous, and the sperm head proceeded slowly but perceptibly towards the egg proper, finally entering the egg cytoplasm, the tail being left in the zona pellucida. I am under the impression that there was a slight bulging out of the egg cytoplasm at the point of entry (cf. Sobotta's description of fertilisation in the mouse, 1895), but as the sperm head enters the cytoplasm it is practically impossible to follow it because of the opacity of the egg cytoplasm. I have, therefore, been unable to ascertain with any exactness the ensuing events. I had thought that if I used ova with practically no adherent follicle cells it would be a comparatively easy matter to observe sperm penetration, but found just the opposite to be the case for two reasons: first, because such ova are rapidly rolled about in culture by the attached spermatozoa; secondly, because if such ova have even the slightest albumen deposit the sperm heads become stuck in the albumen and no further progress is possible. In the case of ova with adherent follicle cells the outgrowth of certain of the follicle cells serves to anchor the eggs on to the culture and they are not rolled about, though they may undergo a certain amount of to and fro movement.

I have in one instance observed the formation of the second polar body, the constriction being completed in about 15 minutes. One observes first a clear area in the cytoplasm at the margin of the egg. This clear area divides into two which separate and gradually disappear. Some time later the polar body is constricted off in the region where one of the two clear areas was observed.

I have also observed what was probably the fusion and subsequent division of the two pronuclei. The ovum in question was observed about 10 hours after insemination. It contained two polar bodies, and towards the centre of the egg and a little to one side was seen a clear area in the cytoplasm, apparently double in nature. This clear area seemed to fuse into a single clear spot, and subsequently divided into two clear areas which moved apart and disappeared. About 3 hours later the egg had divided into two cells.

The writer must apologise for being able to present only these isolated observations. The difficulties encountered in making any accurate observations are, however, considerable. Beside the obscuration of the ova by overlying follicle cells and the movement of the ova themselves, the observer is subjected to a considerable amount of eyestrain which prevents any prolonged



microscopic observation. Furthermore, in a number of instances no development takes place, and I have, on a number of occasions, found myself looking at an egg in which all the significant events had already occurred. It is hoped that by making cinematographic records a fairly complete analysis of the exact sequence of events can be attained.

It is certain that in a number of instances spermatozoa never entered the ova. This is particularly true of the ova in experiment XXIX where a buffered Ringer's solution was the only medium used, where rather careful observation revealed that the adherent follicle cells were quite incompletely removed, and where the sperm died rather rapidly. In other cultures a similar situation probably prevailed. But even in those cases where sperm penetration occurred it is not at all certain that the sperm nucleus was a participant in the segmentation process. The results presented in the preceding section show clearly that segmentation will proceed regularly in unfertilised ova placed in culture. To what extent, therefore, the development observed in the ova of these experiments may be attributable to fertilisation it is impossible to say. When one compares the activity in culture of these ova with those of the same age group that were cultured without spermatozoa we find that of 132 ova placed with sperm 68 divided. If we exclude the ova of experiment XXIX as not comparable we have 68 out of 118, or 60.1 per cent. divided. Ova of the same age group from Table II show 88 out of 136 divided, or 64.7 per cent. Again, of the ova described in Table III (excluding ovarian ova) 50 may be classified as having divided regularly, and 16 as having divided irregularly, or 75.9 per cent. dividing regularly. From the data of Table II we observe that 63 out of 87, or 72.4 per cent. divided regularly in ova of the same age groups without sperm. One may detect from these data no difference between ova cultured with sperm and those without sperm.

To establish the certainty of fertilisation an exhaustive cytological investigation would be necessary, and this is not at present available. I have been able to make an approximate determination of the chromosome number from a division spindle of one ovum into which sperm entry occurred (figs. 36 to 39). There are unquestionably more than the haploid number of chromosomes, but there is no evidence that a sperm contributed any of the chromosomes as diploidy may have arisen by the fusion of two egg nuclei. This is made probable by the presence of a sperm head in the egg cytoplasm and the certain presence of only one polar body. More than one spermatozoon may, however, enter the egg (see fig. 40), and I have in fact observed a pronounced case of polyspermy in one ovum.

The morphological types observed in these experiments are much the same as those observed in ova cultured without sperm. In figs. 41 to 48 I have collected a group of photographs illustrating most of the types observed. Fig. 41 is a photograph of an ovarian ovum placed with sperm and photographed 28 hours after insemination. Fig. 42 represents an ovum recovered at 17 hours and 10 minutes after copulation, and which divided regularly into three cells. Note the sperm clustered about the zona pellucida. Most of these sperm were still alive at 23 hours after insemination. Fig. 43 represents an ovum in which one of the early blastomeres remained quiescent while the others proceeded to divide regularly. The ovum of fig. 44 apparently divided quite regularly and it contains obviously two polar bodies. The succeeding figures (figs. 45 to 48) represent the more irregular types of division observed.

My observations indicate that sperm may penetrate the ovum as long as it is not surrounded by albumen. The ovum of fig. 36 was recovered as late as 17 hours and 10 minutes after copulation practically free of adherent follicle cells and sperm entry undoubtedly occurred *in vitro*. This finding is of much interest in connection with recent studies (Hammond and Marshall, 1925; Hammond, 1928) on the length of time the rabbit ovum is fertilisable *in vivo*. Using the ingenious method of mating rabbit does first to vasectomised bucks to ensure ovulation, and then to fertile bucks at various intervals after the sterile mating, it was demonstrated that when the fertile mating is made 14 hours after the sterile mating no progeny are obtained. Furthermore, when fertile matings are made during the period between 6 and 12 hours after the sterile mating there occurs a fairly regular decrease both in the percentage of does producing young, and in the average litter size. Assuming that ovulation takes place at 10 hours after the first copulation, and that it takes the sperm about 4 hours to reach the tops of the tubes Hammond concludes that the ova are fertilisable for at most 6 hours after ovulation and ordinarily for not so long a period.

It seemed just possible that the unproductive ova may have been fertilised in Hammond's experiments, but that development was initiated too late to permit normal implantation. I accordingly mated a doe to sterile bucks and 14 hours later to fertile bucks. Twenty-nine hours and 10 minutes later, or 43 hours and 10 minutes after the sterile copulation, the doe was killed and eight ova were recovered. If fertilisation and segmentation had occurred the ova should have been in at least four cells. They were actually all in one cell, and a careful examination of the zona pellucida revealed no sign of sperm heads such as are seen in normally fertilised ova. The obvious conclusion is

that sperm penetration did not take place. With another doe I repeated the mating described, but killed the animal and recovered the ova at 3 hours 40 minutes after the fertile mating or 17 hours and 40 minutes after the sterile mating. Eight ova were recovered, all of them separated out of the cumulus mass but still surrounded by a number of follicle cells. The ova were typical of eggs recovered from sterile matings at the same time, and the photograph of one of them is given in fig. 49. Many sperm were detected in the washing fluid, but none in the zona pellucida of any ovum or in the adherent follicle cells. A third mating was made in which the fertile mating was conducted 12 hours after the sterile mating. The doe was killed at 8 hours and 10 minutes after the fertile mating, or 20 hours and 10 minutes after the sterile mating, and two ova were recovered. The zona pellucida of one ovum was entirely free of sperm heads, while in the zona pellucida of the second ovum a single sperm head was detected. It should be noted that from such matings Hammond rarely obtained litters.

The obvious conclusion from these experiments is that the sterility resulting from this type of mating is due to the fact that sperm do not enter the egg. There is no reason for suspecting that the ovum itself prevents sperm entry after a definite time. If this were the case it is hard to understand why so many sperm are seen in the zona pellucida of a normally fertilised ovum. It has been pointed out that the position of many sperms in the zona pellucida indicates that the fertilising spermatozoon is probably one which enters the zona head on. Since such a type of entry is accidental it follows according to the laws of chance that ordinarily a number of sperm must attach themselves to the egg. If a variation from the perpendicular of as much as 10 degrees prevents complete entry of the sperm head then it follows that one sperm in 18 will enter at the correct angle. This is taken to imply that it is not necessarily the first sperm to reach the egg that accomplishes fertilisation, but rather one of the main body of sperm.

Now Hammond bases his calculations as to the length of time the egg is fertilisable on data from Heape (1905), which indicates that a few sperm can be detected at the tops of the tubes by 4 hours after copulation. Lloyd-Jones and Hays (1918) calculating from the rate of motion of sperm *in vitro*, arrive at the conclusion that the sperm reach the tops of the tubes in about 3 hours, but their calculations are admittedly rough. From these fragmentary observations it is impossible to determine exactly when the main body of sperm reach the tops of the tubes, and it is probably a spermatozoon from the main body of the sperm which is concerned in the fertilisation process. If we take Heape's

estimate of 4 hours as correct, then it follows that 6 hours after ovulation is the latest time at which fertilisation is possible. I have shown that at this time the ova from sterile matings are for the most part in the cumulus mass, the process of detachment having just begun. Since it has been shown that it is improbable that the ova themselves are responsible for the non-entry of sperm it follows that either (1) some process going on in the tubes is responsible for non-entry, or (2) the sperm have not reached the eggs.

If we consider the first possibility there is this explanation, namely, that sperm reaching the cumulus mass at 6 hours after ovulation begin the process of penetrating the surrounding follicle cells. This process takes on the average 2 hours. At the same time the ova are spontaneously separating out, by 18 hours after copulation, or 8 hours after ovulation, practically all ova are separated out of the granulosa mass. Can it be that the ova that separate out pass so rapidly through the main body of sperm that it is impossible for sufficient sperm to attach themselves to these ova to make the chances of penetration likely? The fact that an occasional ovum does remain in the cumulus mass until this time may account for the occasional litter obtained by Hammond in such a mating.

The other possibility is that the main body of sperm reaches the tops of the tubes not at 4 hours after the fertile copulation but some time later. In this case the sperm from fertile matings made some time after the sterile mating might arrive during the period when ova are becoming detached from the cumulus mass and the number of ova fertilised would depend on the number of ova without any albumen coating. Thus, during the period from 15 to 20 hours after sterile coition progressively more ova become covered with albumen, and it has been demonstrated that sperm cannot penetrate the albumen coating. For the exact coincidence of the period when ova are separating out of the cumulus mass and the arrival of the spermatozoa much more than 4 hours would be required for the ascent of the sperm to the tops of the tubes. My observations indicate that sperm are certainly present in some part of the tubes in large numbers by 3 hours and 40 minutes after a fertile copulation.

It is obvious that more information than is at present available is needed for ascertaining just why the ova remain fertilisable for such a short time. An exhaustive investigation would certainly be worth while in view of the pertinence of the ensuing results to the general problem of the fertilising capacity of the ovum.

Attempts have been made to transplant culture-grown ova from the culture into the Fallopian tubes of pseudo-pregnant rabbit does. No offspring have

been recovered for a number of reasons, chiefly technical. Figs. 50 and 51 illustrate the difficulties of the technique of picking up the ova in a pipette and injecting them through the fimbriated end of the tubes.\* The ovum of fig. 50 was injected after 26 hours of culturing without sperm. It was in four regular cells when injected and evidently continued to develop to some extent during the 50½ hours it was in the animal. It was recovered from the uterus and was evidently degenerating. Note the presence of many air bubbles in the albumen coating, never observed in normal ova. It is probable that the ovum entered the uterus before it was prepared for the growth stage and consequently degenerated. This is certainly the case with the ovum of fig. 51 which was placed in culture with sperm for 25 hours and recovered from the uterus 24 hours later. Note the very slight albumen coating and obvious degeneration. It was probably injected too far down the tubes and entered the uterus much too soon. It is hoped that technical refinements will permit successful transplantation. Even with normally fertilised ova obtained directly from the tubes of one doe and transplanted into the tubes of another pseudopregnant doe of which three experiments were made as controls, only one doe produced a litter (of three young) at the thirty-first day. Experiments previously made by Adsell and Hammond (but not yet published) also show a very low yield of successes, only three does out of 21 such transplantations made, giving litters of young; the young produced, however, were quite normal. Technical refinements in the method of transplantation are therefore required before further attempts are made to rear the incubated eggs by transplantation.

The writer wishes to acknowledge with gratitude the very kind treatment and co-operation accorded him by Dr. F. H. A. Marshall, Mr. John Hammond, and Dr. Arthur Walton of the Laboratory of Animal Nutrition, The School of Agriculture, Cambridge University. He is also indebted to Dr. Honor B. Fell for putting the resources of the Strangeways Research Laboratory at his disposal, and to Dr. R. G. Canti of St. Bartholomew's Hospital, London, for his invaluable co-operation in the difficult task of filming developing ova. The results here presented were obtained during a 7 months' stay at Cambridge University, and the work was carried on chiefly under the direction of Mr. John Hammond.

\* Operations were carried on by Mr. John Hammond and Dr. Arthur Walton of the School of Agriculture, Cambridge University.

*Summary.*

1. Ova from rabbit does mated to vasectomised bucks are liberated from the ovary surrounded by cumulus cells. They become massed together in the upper part of the tubes and remain in this mass until about 17 hours after copulation when they begin, gradually, to separate out.

2. By 20 hours after copulation practically all unfertilised ova are separated out of the cumulus mass, and are covered with a thin coating of albumen.

3. Subsequently, the ova descend the tubes, becoming covered with a thick albumen deposit. They enter the uterus between 72 and 96 hours after copulation where no further albumen deposit occurs and marked degeneration is observable. Uterine ova soon disappear.

4. Except in three instances where, apparently, irregular fragmentation occurred all ova from matings to vasectomised bucks were in the one cell stage when recovered.

5. Sperm heads were never detected in the zona pellucida of ova recovered from does mated to vasectomised bucks. Such ova did, however, occasionally contain two or even three polar bodies.

6. Ova from matings to fertile bucks also emerge from the ovary surrounded by cumulus cells, but remain massed together in the tubes for only 1 to 1½ hours after ovulation. The detachment of the surrounding follicle cells is evidently brought about by the penetration of sperm.

7. By 14 hours after copulation, or 4 hours after ovulation, all ova from fertile matings are practically completely free of adherent follicle cells and sperm entrance has taken place.

8. By 17 hours after copulation the second polar body is formed in fertilised ova, and by 18 hours after copulation the separation of the first segmentation nuclei can be observed. The first segmentation division occurs at about 21 hours after copulation.

9. The proportion of ova from fertile matings that divide in culture is very low for ova recovered between 11 and 12 hours after copulation, increases for ova obtained from 12 to 13 hours after copulation, and practically all ova recovered from 14 hours after copulation onwards divide in culture. This is taken to indicate that the fertilising spermatozoon enters the egg between 12 and 13 hours after copulation, or in 2 to 3 hours after ovulation.

10. Almost all one-celled ova from fertile matings dividing in culture segment with great regularity.

11. One-celled fertilised ova placed in culture may develop as far as the

morula, but no attempt has been made in these experiments to prolong the life of rabbit ova *in vitro*.

12. Over 60 per cent. of unfertilised ova placed in culture without sperm undergo development *in vitro*. The regularity of the development in certain cases and its similarity to the development of fertilised ova justifies the appellation of parthenogenesis.

13. Irregular development occurs principally in the older tubal ova and probably consists of a degenerative fragmentation. The younger tubal ova (recovered from 11 to 20 hours after copulation) are usually truly parthenogenetic in that the egg nucleus (or nuclei) participate in the regular segmentations observed. It is suggested that irregular development is correlated with the degree of cytoplasmic degeneration of the ova.

14. Development *in vitro* may be due (1) to sudden temperature change, (2) to an increase in oxygen tension and decrease in carbon dioxide tension, or (3) to a gradually developed hypertonicity of the medium. It is suggested that the culture medium employed nourishes the ova, and is probably not a stimulant to development.

15. The actual process of segmentation in fertilised and unfertilised ova takes 9 to 10 minutes and ordinarily there are formed two slightly unequal cells at the first segmentation division. Fertilised ova recovered soon after fertilisation begin to segment later than those recovered some time after fertilisation. Unfertilised ova ordinarily begin to segment at from 6 to 14 hours after culturing or as late as 26 hours after culturing. Older (fragmenting?) ova may begin division at once.

16. Two ova have been observed to begin segmentation and then to reverse this process after exposure to room temperature.

17. Sperm entry undoubtedly occurs in ova placed with sperm *in vitro* provided these ova have no albumen coating. It is not certain that the sperm nucleus enters into the subsequent mitoses even though the ova are diploid.

18. Semination *in vitro* resembles the same process *in vivo* in that the sperm must penetrate the adherent follicle cells before they can reach the eggs. This process *in vitro* ordinarily takes from 2 to 3 hours and a similar time is required *in vivo*.

19. In matings in which a copulation with a sterile buck is followed 14 hours later by copulation with a fertile buck no young are produced because no sperm enter the ova. This is probably due to the fact that an insufficient number of sperm (or no sperm?) reach the ova to ensure sperm entry before the ova become covered with albumen and therefore impenetrable.

20. Attempts to transplant culture grown ova into rabbit does have been made, but no young were ever produced. Sufficient technical refinement may, however, permit successful transplantations, as indicated by the result of one normal transplantation experiment.

# EXPLANATION OF PLATES 10-13.

## PLATE 10.

- FIG. 1.—Three ova in the cumulus mass recovered from the Fallopian tubes of rabbit doe 12½ hours after a sterile mating.
- FIG. 2.—An unfertilised ovum still in the cumulus mass 16 hours after a sterile mating.
- FIG. 3.—Another 16-hour ovum free of the cumulus mass.
- FIG. 4.—An ovum recovered 19 hours and 5 minutes after a sterile mating with no adherent follicle cells.
- FIG. 5.—An ovum recovered 24½ hours after a sterile mating showing a definite albumen coating.
- FIGS. 6 to 9.—All from sterile matings at the following intervals after sterile copulation :— 6, 43 hours 30 minutes ; 7, 73 hours 40 minutes ; 8 and 9, 96 hours 45 minutes.
- FIG. 10.—An ovum recovered 41 hours and 30 minutes after a sterile copulation which developed " many polar bodies " *in vitro* (see text).
- FIGS. 11 and 12.—Ova recovered from fertile matings at 13 hours and 18 hours and 50 minutes after copulation respectively (note second polar body and albumen coating of ovum of fig. 12).

## PLATE 11.

- FIGS. 13 to 15.—Ova recovered from fertile matings at 20 hours and 30 minutes, 27 hours and 15 minutes, and 37 hours after copulation respectively.
- FIG. 16.—Cinematographic record of the first segmentation division *in vitro* of a fertilised ovum recovered at 14 hours and 15 minutes after copulation. (Note sperm head in zona pellucida at lower right.)
- FIG. 17.—Cinematograph record of the first segmentation division *in vitro* of an unfertilised ovum recovered at 19½ hours after sterile copulation.
- FIGS. 18 to 24.—Ova from sterile matings as they appeared after being cultured *in vitro*.
- FIG. 18.—Recovered at 18 hours 30 minutes after sterile copulation cultured for 44 hours.
- FIG. 19.—Recovered at 27 hours 30 minutes after sterile copulation cultured for 25 hours.
- FIG. 20.—Recovered at 19 hours 5 minutes after sterile copulation cultured for 22 hours.
- FIG. 21.—Recovered at 18 hours 10 minutes after sterile copulation cultured for 17 hours.
- FIG. 22.—Recovered at 18 hours 15 minutes after sterile copulation cultured for 28 hours.
- FIG. 23.—Recovered at 19 hours 5 minutes after sterile copulation cultured for 22 hours.
- FIG. 24.—Recovered at 19 hours 5 minutes after sterile copulation cultured for 22 hours.



## PLATE 12.

**Figs. 25 to 32.**—Ova from sterile matings as they appeared after being cultured *in vitro*.

**FIG. 25.**—Recovered at 28 hours 35 minutes after sterile copulation cultured for 23 hours.

**FIG. 26.**—Recovered at 17 hours 10 minutes after sterile copulation cultured for 23 hours.

**FIG. 27.**—Recovered at 24 hours 45 minutes after sterile copulation cultured for 24 hours.

**FIG. 28.**—Recovered at 37 hours after sterile copulation cultured for 6 hours.

**FIG. 29.**—Recovered at 73 hours 40 minutes after sterile copulation cultured for 45 hours.

**FIG. 30.**—Recovered at 73 hours 40 minutes after sterile copulation cultured for 45 hours.

**FIG. 31.**—Recovered from the ovary, and cultured for 28 hours.

**FIG. 32.**—Recovered at 48 hours 30 minutes after sterile copulation cultured for 24 hours.

**FIG. 33.**—Section of four-celled parthenogenetic ovum recovered at 15 hours after sterile copulation and cultured for 29 hours. Fixed in Bouin's solution, stained with iron-hematoxylin.

**Figs. 34 and 35.**—An ovum recovered in the one-cell stage at 5.15 p.m. 41 hours 30 minutes after a sterile copulation, incubated till 7.35 p.m. (see clock) removed to room, then photographed (at 7.40) in cinematographic incubator. Second photograph (at 8.13 p.m.) indicates reversal of initiated segmentation.

**FIG. 36.**—Section showing part of spindle of ovum recovered at 17 hours 10 minutes after sterile copulation and placed in culture with sperm for 23 hours. Fixed in Bouin-Allen fluid, stained with iron-hematoxylin. (Note sperm head between polar body and spindle.)

**FIG. 37.**—Analysis of spindle of fig. 36.

**FIG. 38.**—The other half of the spindle of the ovum of fig. 36.

## PLATE 13.

**FIG. 39.**—Analysis of spindle of fig. 38.

**FIG. 40.**—Section of ovum recovered at 11 hours after sterile copulation, and cultured with sperm for 28 hours. Note two sperm heads in cytoplasm and two nuclei (no cell divisions). Fixed in acetic alcohol, stained with hematoxylin.

**Figs. 41 to 48.**—Ova from sterile matings and placed in culture with sperm.

**FIG. 41.**—Ovarian ovum cultured for 28 hours. (Note outgrowth of follicle cells.)

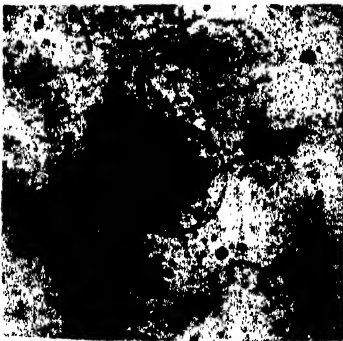
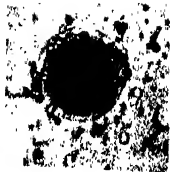
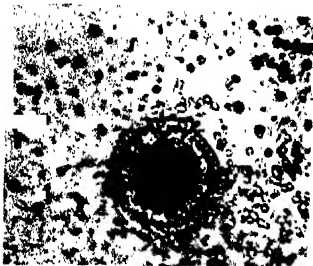
**FIG. 42.**—Ovum recovered at 17 hours 10 minutes after sterile copulation, cultured for 23 hours.

**FIG. 43.**—Ovum recovered at 18 hours 10 minutes after sterile copulation, cultured for 26 hours.

**FIG. 44.**—Ovum recovered at 14 hours 1 minute after sterile copulation, cultured for 26 hours.

**FIG. 45.**—Ovum recovered at 16 hours 10 minutes after sterile copulation, cultured for 26 hours.

**Figs. 46 to 48.**—Ova recovered at 15 hours 15 minutes after sterile copulation, cultured for 35 hours.



7



8



9



10



11



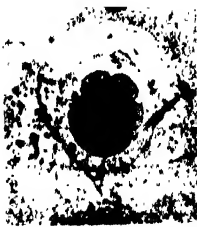
12



13



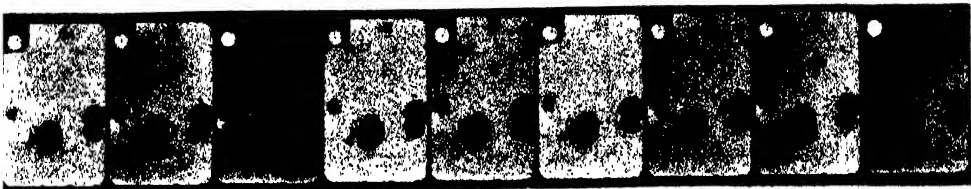
14



15



16



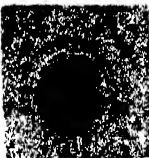
17



18



19



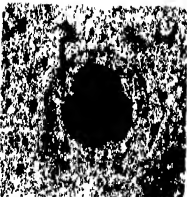
20



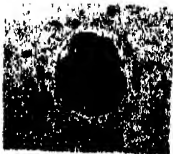
21



22



23



24



25



26



27



28



29



30



31



32



33



34



35



36



37



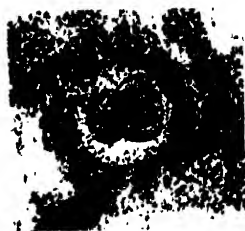
38



39



40



41



42



43



44



45



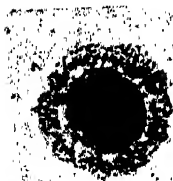
46



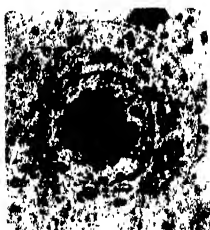
47



48



49



50



51

- FIG. 49.—Ovum recovered 17 hours 40 minutes after sterile copulation and 3 hours 40 minutes after (a second) fertile copulation.
- FIG. 50.—Ovum recovered from uterus 50 hours 30 minutes after it had been injected into the Fallopian tubes. This ovum was originally recovered at 19 hours 30 minutes after a sterile copulation and was in four cells after 26 hours of culturing *in vitro* before transplantation.
- FIG. 51.—Ovum recovered from uterus 24 hours after it had been injected into the Fallopian tubes. It was obtained from the ovary, placed *in vitro* with sperm for 25 hours, and when transplanted had not segmented.

#### REFERENCES.

- Asami, G. (1920). 'Anat. Rec.,' vol. 18, p. 323.
- Assheton, R. (1894). 'Quart. J. Micr. Sci.,' vol. 37, p. 113.
- Branca, A. (1925). 'Arch. Biol.,' vol. 35, p. 325.
- Campbell, J. A. (1924). 'J. Physiol.,' vol. 59, p. 1.
- Champy, C. (1927). 'C. R. Soc. Biol.,' vol. 96, p. 1108.
- Charlton, H. H. (1917). 'Biol. Bull.,' vol. 33, p. 321.
- Hammond, J. (1928). 'Z. Züchtungsk.,' vol. 3, p. 523.
- Hammond, J., and Marshall, F. H. A. (1925). "Reproduction in the Rabbit," Edinburgh.
- Hartman, C. G. (1926). 'Amer. J. Anat.,' vol. 37, p. 1.
- Heape, W. (1905). 'Proc. Roy. Soc.,' B, vol. 76, p. 1.
- Hindle, E. (1910). 'Arch. Entw. Mech.,' vol. 31, p. 145.
- Just, E. E. (1928). 'Biol. Bull.,' vol. 55, p. 358.
- Kampmeier, Otto F. (1929). 'Amer. J. Anat.,' vol. 43, p. 45.
- Lewis, W. H., and Gregory, P. W. (1929, a). 'Science,' vol. 69, p. 226.
- Lewis, W. H., and Gregory, P. W. (1929, b). 'Anat. Rec.,' vol. 42, p. 27.
- Lloyd-Jones, O., and Hays, F. A. (1918). 'J. Exp. Zool.,' vol. 25, p. 463.
- Loeb, Jacques (1913). "Artificial Parthenogenesis and Respiration," Chicago.
- Long, J. A. (1912). 'Univ. Calif. Publ. Zool.,' vol. 9, p. 105.
- Long, J. A., and Evans, H. M. (1922). 'Mem. Univ. Calif.,' vol. 6.
- Morris, Margaret (1917). 'J. Exp. Zool.,' vol. 22, p. 1.
- Pannett, Charles A., and Compton, Arthur (1924). 'Lancet,' p. 381.
- Sansom, G. S. (1920). 'J. Anat.,' vol. 55, p. 68.
- Scott, J. (1906). 'J. Exp. Zool.,' vol. 3, p. 49.
- Sobotta, J. (1895). 'Arch. Mikr. Anat.,' vol. 45, p. 15.
- Walton, A., and Hammond, J. (1928). 'Brit. J. Exp. Biol.,' vol. 6, p. 190.
- Wilson, E. B. (1901). 'Arch. Entw. Mech.,' vol. 12.

## PENDIX

TABLE 1.—THE BEHAVIOR OF THE OVA FROM DOES MATED TO FERTILE DUCKS.

(C = Chicken. R = Rabbit. P = Plasma. E = Embryo extract.)

	Age of Ova (hours after copulation).	Number.	Medium.	Examined (hours in culture).	Description.	Number divided.	Number undivided.	Experiment No.	Remarks.
(1)	h. m. 11 0	6	CPCE	50	4—unsegmented ..... 1—in 8 equal cells ..... 1—in 2 unequal cells .....	2	4	XI	Ova heavily surrounded by follicle cells but separate. From small doe.
(2)	11 1	5	Ringer's	48	5—unsegmented .....	0	5	XXIX	Three in cumulus mass and two separate but heavily surrounded by cumulus cells.
(3)	11 38	6	Ringer's	26	6—unsegmented .....	0	6	XXIX	Ova same as in (1).
(4)	11 55	6	CPCE	44	1—in 4 equal cells ..... 1—in 2 unequal cells .....	2	4	VIII	Adherent cells less nu- merous than in (1).
(5)	12 20	8	Ringer's	22	4—unsegmented ..... 3—2 equal cells ..... 3—3 to 4 cells ..... 2—unsegmented .....	6	2	XXIX	Adherent cells much re- duced with slight areas quite free of any fol- licle cells in most ova.
(6)	13 0	8	RCPCF	49	1—4 regular cells ..... 2—4 regular cells (infected) 2—8 regular cells ..... 3—unsegmented ..... 1—2 regular cells ..... 1—16 regular cells .....	5	3	XIX	Ova as in (5). Large doe.
(7)	13 7	3	CPCE	50	1—unsegmented ..... 3—in 3 regular cells ..... 1—in 4 regular cells .....	2	1	XI	Ova as in (5). Large doe.
(8)	14 15	4	RPRE	37	1—unsegmented ..... 1—in 4 regular cells .....	4	0	L	Ova all clear of adherent cells but no albumen deposit.
(9)	14 30	3	CPCE	54	3—in 8 to 16 regular cells .....	3	0	XXIX	Ova as in (8).

(10)	14	30	4	Various	48	4—unsegmented	XXIX	2 ova in hanging drop culture. 1 in chick embryo extract in sealed slide; 1 on plasma-extract clot in sealed slide; 2 nuclei observed, but no segmentation.
(11)	15	0	6	Ringer's	48	5—in 4 regular cells 1—unsegmented		Unsegmented ovum in hanging drop showed 2 nuclei but no segmentation. All ova free of adherent cells.
(12)	15	0	6	CPCE	45	6—in morula	XII	Ova free of adherent cells and with slight albumen deposit.
(13)	15	0	3	RPCE	25	1—in 3 large and some small cells	XIX	Ova as in (12).
(14)	15	10	7	RPCE	25	2—4 to 6 regular cells 2—in 2 cells (infected) 4—4 regular cells	XXII	Ova as in (12) except one still surrounded by follicle cells.
(15)	15	20	2	RPCE	26	1—unsegmented but with 6 to 7 polar bodies		Ova as in (12).
(16)	16	0	6	CPCE	45	2—6 regular cells 6—in 8 regular cells	X	Obvious but slight albumen deposit about the eggs.
(17)	17	20	6	RPCE	23	2—in 2 to 3 cells regular		Albumen deposit slightly greater than in (16).
(18)	18	15	8	RPCE	30	4—in 8 to 16 regular cells 1—in 4 regular cells 1—in 4 unequal cells 1—in 8 regular cells 2—in 10 regular cells		
(19)	20	30	2	RPCE	24	2—in 8 regular cells		
(20)	21	20	5	CE	24	4—in 4 to 8 regular cells 1—in many irregular cells	X	
(21)	27	55	7	RPCE	24	Shrunk and degenerate but in 5-6 cells regular		Six other ova from the same doe in 2 cells but not cultured.
(22)	37	0	4	BPCE	48	3—in morula	XIV	Ova in 2, 3 and 4 cells. Much albumen deposit.
(23)	45	0	3	RPCE	26	1—in 24 to 32 cells	XIX	Ova in 8 to 9 cells.
(24)	49	45	7	4—in RPCE 3—in CPCE	46	3—in 36 to 48 regular cells	XVIII XIII	Ova in 10 to 16 cells.
(25)	69	0	7	4—in RPCE 3—in CPCE	46	2—in gastrula 5—in gastrula 2—in morula	XIII	Ova in 16 to 20 cells.

in morula. All in RPCE gastrulated. One ovum in RE gastrulated.



TABLE 11.—THE BEHAVIOR *IN VITRO* OF OVA FROM DOES MATED TO STERILE BUCKS.  
(C = Chicken. R = Rabbit. P = Plasma. E = Embryo extract.

	Age of Ova (hours after ovulation).	Number.	Medium.	Examined (hours in culture).	Description.	Number divided.	Number undivided.	Experiment No.	Remarks.
(1)	0 0	1	RPPE	44	1—unsegmented	0	1	XVIII	
(2)	0 0	4	RPCE	48	3—unsegmented	1	3	XXVII	
(3)	0 0	4	RPCE	48	1—in 3 regular cells				
(4)	11 9	1	CPCE	48	4—unsegmented	0	4	XXVIII	
(5)	11 25	5	RPPE	24	1—several polar bodies ?	1 (?)	0 (?)	IX	
					3—unsegmented	2	3	XV	
(6)	11 40	2	RPPE	48	1—8 regular cells				
(7)	12 5	5	RPPE	47	1—4 regular cells	0	2	XV	
					2—unsegmented	2	3	XIII	
(8)	12 30	6	RPCE	27	2—in 12 to 16 regular cells				
					1—in 3 regular cells	6	0	XIX	
					1—in 3 regular cells				
					1—in 4 regular cells				
					3—in 5 to 6 regular cells				
(9)	13 15	3	RPCE	47	2—unsegmented	1	2	XX	
					1—16 to 20 cells				
(10)	13 35	11	RPPE	45	1—in 2 cells and 2 to 5 polar bodies	4	7	XVIII	
					3—with about 5 polar bodies				
					7—unsegmented				
(11)	13 50	4	RPPE	48	2—unsegmented	2	2	XVII	
					1—in 4 regular cells and 2 polar bodies				
					1—in morula				
(12)	14 1	1	RPPE	25	1—unsegmented	0	1	XVI	
(13)	14 35	6	RPCE	27	2—in 3 regular cells	6	0	XXIII	
					2—in 4 regular cells				
					2—in 36 to 40 regular cells				
					2—in about 4 cells regular				
(14)	15 0	3	CPCE	30	1—with multiple polar bodies	3	0	XII	

5)	15 15	3	RPRE	27	1—unsegmented 1—in 4 regular cells 1—in about 16 cells 2—in 1 very large cell and 10 to 12 small ones 2—in about 16 cells 1—in 32 to 48 cells 1—in 2 regular cells and 2 polar bodies 1—in 3 to 4 large cells and 10 small cells 1—in 1 large cell and 16 small cells	2	1	XVI	Incomplete metaphase plate of one ovum showed at least 37 chromosomes.
6)	16 0	5	RPCE	22	2—no segmentation 1—2 large, 2 small cells and several polar bodies 1—in 16 very regular cells 2—in 20 to 32 cells 3—unsegmented 1—2 unequal cells and 7 to 8 polar bodies 1—3 cells and several polar bodies 1—4 regular cells 1—in 10 small cells and 1 large cell	0	2	IX XXVII	
7)	17 10	3	RPCE	23	4—unsegmented 1—3 cells regular 2—about 4 regular cells, but shrunk 1—in 12 regular cells 1—in about 8 cells, but shrunk 1—unsegmented 1—3 polar bodies 2—unsegmented 1—1 large cell and 2 to 3 small cells 1—2 regular cells and 2 polar bodies 1—4 regular cells 1—7 regular cells 4—about 8 cells	3	0	XXVIII	
	17 33 17 45	2 4	CPCE RPCE	48 26		4	3	XX	
	18 10	7	RPCE	48		5	4	XXVI	5 ova 3 in the clot, and 2 on the clot.
	18 15	9	RPCE	29		1 (1)	1	XI	3 ova in plasma alone divided but shrunken; 3 ova in the clot, 1 divided; 3 ova on the clot, 1 divided.
	18 20	2	CPCE	47		8	2	XXV	3 ova were on a clot formed in a hollow ground slide and they all segmented.
	19 25	10	RPCE	24					

Table continued).  
 C Chicken R Rabbit = Plasma. E bryo extract.

Age of Ova (hours after copulation).	Number	Examination (hours in culture)	Observation.	Number undivided	Experiment No.	Remarks
24	8 30	RPRE	48		X	inf.
25	8 50	RPRE	2—unsegmented 1—in 3 cells 2—in 8 regular cells		X	
	9 5	RPCE	22		XX	3 ova in segmented
			1—in 10 regular cells 2—unsegmented 2—in 2 regular cells 4—in 4 regular cells 1—in 7 cells 1—2 unequal cells and 3 polar bodies			
		RPCE	1—unsegmented 1—2 regular cells 2—4 regular cells		XX	
26	45	RPCE	1—unsegmented 3—in 2 cells 2—in 4 cells 1—in 6 cells 1—in 8 cells 2—many polar bodies 3—in many cells 4—unsegmented 1—unsegmented 1—1 large and 2 to 3 small cells		XX	3 ova in plasma only seg- mented; 2 ova in clot segmented; 2 out ova on clot segmented.
	20 0	CPCE			VI	
	20 10	RPRE			XVI	
	20 20	RPCE			XX	
	20 20	CPCE			XII	
		RPCE	1—2 unequal cells 3—2 regular cells 1—3 regular cells 1—1 large cell and several polar bodies		XX	ova in embi alone seg in fection.
24 45		RPRE	1—16 to 20 regular cells and a few polar bodies		X	

(35)	27	35	5	CPCE	46	1—unsegmented 2—about 8 cells and many polar bodies 2—one large cell and many polar bodies 4—unsegmented 1—4 regular cells 1—6 unequal cells 3—about 8 cells 2—in many cells and degenerate 3—in many small cells	4	1	VII	Sperm placed in culture but entry impossible because of albumen layer.
(36)	28	35	9	RPCE	47	4—unsegmented 1—4 regular cells 1—6 unequal cells 3—about 8 cells 2—in many cells and degenerate 3—in many small cells	5	4	XXVII	2 ova in embryo; extract only did not segment. One ovum in clot segmented.
(37)	37	30	2	ROPCE	27	2—in many cells and degenerate 3—in many small cells	2	0	XIX	
(38)	40	40	3	CPCE	45	3—in many small cells	3	0	VII	Sperm placed with two ova but albumen prevented penetration.
(39)	43	10	6	CPCE	46	1—in 2 cells 1—in 4 cells and many polar bodies 2—in 1 cell and many polar bodies 2—in many small cells 3—unsegmented 1—in 2 unequal cells 2—with many polar bodies 5—unsegmented 3—with many polar bodies	6	0	XI	Doe mated to fertile bucks 14 hours after sterile copulation. All ova in one cell when recovered.
(40)	47	30	6	RPCE	22	2—in many small cells 3—unsegmented 1—in 2 unequal cells 2—with many polar bodies 5—unsegmented 3—with many polar bodies	3	3	XXVIII	
(41)	48	30	8	RPCE	48	1—in 2 unequal cells 2—with many polar bodies 5—unsegmented 3—with many polar bodies	3	5	XXVII	3 ova in plasma only unsegmented; 2 ova in clot unsegmented.
(42)	48	47	6	RPRE	52	1—about 8 large cells 1—3 unequal cells and many polar bodies 4—with many polar bodies 1—unsegmented 4—in many cells 3—unsegmented and degenerate 2—unsegmented and shrunken 2—about 10 polar bodies and shrunken 4—unsegmented 2—16 regular (?) cells 1—5 cells and 3 polar bodies 2—unsegmented	6	0	XIII	4 ova with sperm but no penetration through albumen layer.
(43)	50	30	5	RPRE	45	4—with many polar bodies 1—unsegmented 4—in many cells 3—unsegmented and degenerate 2—unsegmented and shrunken 2—about 10 polar bodies and shrunken 4—unsegmented 2—16 regular (?) cells 1—5 cells and 3 polar bodies 2—unsegmented	4	1	XVIII	Ova with sperm but no penetration.
(44)	68	33	3	RPRE	45	3—unsegmented and degenerate 2—unsegmented and shrunken 2—about 10 polar bodies and shrunken 4—unsegmented 2—16 regular (?) cells 1—5 cells and 3 polar bodies 2—unsegmented	0	3	XIII	2 ova in plasma only segmented.
(45)	72	0	4	RPCE	22	2—unsegmented and shrunken 2—about 10 polar bodies and shrunken 4—unsegmented 2—16 regular (?) cells 1—5 cells and 3 polar bodies 2—unsegmented	2	2	XXVI	
(46)	73	40	7	RPCE	45	4—unsegmented 2—16 regular (?) cells 1—5 cells and 3 polar bodies 2—unsegmented	3	4	XX	
(47)	96	45	2	RPCE	46	2—unsegmented	0	2	XX	

Table III.—Data on Attempts at Fertilisation *in vitro*.  
(C = Chicken. R = Rabbit. P = Plasma. E = Embryo extract.)

	Age of Ova (hours after copulation).	Number.	Medium.	Examined (hours in culture).	Description.	Number divided.	Number undivided.	Experiment No.	Remarks.
(1)	0 0	8	RPRE	44	1—in 3 cells 1—with many polar bodies 6—unsegmented 7—unsegmented	2	6	XVIII	Ova from unburst follicles 11 hours after sterile copu- lation.
(2)	0 0	7	CPCE	48		0	7	III	
(3)	0 0	5	RPCE	48	5—unsegmented	0	5	XXVII	
(4)	11 0	3	Ringer's	48	3—unsegmented	0	3	XXIX	After 24 hours 2 ova still sur- rounded by follicle cells, one fairly free of follicle cells, 3 ova were inseminated in liquid medium.
(5)	11 0 to 11 15	6	CPCE	48	1—unsegmented 2—2 regular cells 2—3 to 4 regular cells 1—6 cells	5	1	IV	
(6)	11 7	3	CPCE	48	1—in 2 regular cells 2—in 3 regular cells	3	0	IX	
(7)	11 20	1	CPCE	48	1—in 2 to 4 cells	1	0	III	
(8)	11 27	8	CPCE	48	5—unsegmented 3—with many polar bodies	3	5	II	
(9)	11 30	3	CPCE	49	1—unsegmented 1—in 4 regular cells 1—in about 8 cells 4—unsegmented	2	1	IX	Sperm poor, penetration im- probable.
(10)	11 32	4	Ringer's	24		0	4	XXIX	Cumulus mass unbroken, probably no penetration. Follicle cells as in (4).
(11)	11 45	5	Ringer's	48	5—unsegmented	0	5	XXIX	
(12)	12 5	3	RPRE	47	2—2 cells	3	0	XIII	
(13)	12 8	3	CPCE	52	1—4 cells 1—in 2 regular cells 2—with many polar bodies	3	0	I	
(14)	12 30	2	RCPE	27	1—unsegmented 1—in 6 cells	1	1	XIX	Sperm seen in zona pellucida.

[5]	13	0	3	RPCE	46	1—unsegmented 1—partially segmented 1—in 2 regular cells 3—unsegmented 1—3 to 4 polar bodies 1—2 to 4 cells 1—in 8 regular cells 5—unsegmented 3—unsegmented 3—in 4 to 8 cells 1—in 6 to 7 cells 2—in 2 regular cells 1—in 4 regular cells 1—with many polar bodies 2—unsegmented 1—in 4 cells 4—unsegmented 1—unsegmented 1—many cells 1—1 large cell and 8 to 10 small cells 1—1 large cell and several small ones 1—2 regular cells and 2 to 4 polar bodies 2—in many cells 1—unsegmented 1—1 large and 3 to 4 small cells 1—2 cells 1—7 unequal cells 1—many cells 1—2 cells 1—4 cells 1—8 cells 6—in 10 to 24 cells 2—unsegmented 2—in 6 to 8 regular cells 4—in 2 to 6 cells 1—unsegmented 2—in 2 to 3 cells 1—in 2 cells and several polar bodies 2—many polar bodies 1—unsegmented 1—in 4 to 8 cells 2—unsegmented	2 (?)	1	Infected.
[6]	13	15	6	RPCE	47		3 (?)		XX
(17)	13	25	5	RPCE	27		0	5	XVII
(18)	13	50	3	RPCE	48		0	3	XVII
(19)	14	1	4	RPCE	25		4	0	XVI
(20)	14	35	2	RPCE	27		2	0	XVIII
(21)	15	0	4	CPCE	46		2	2	VI
(22)	15	0	5	CPCE	46		1	4	XII
(23)	15	15	3	RPCE	48		2	1	XVI
	16	0	4	RPCE	22		4	0	XXVIII
	17	10	5	RPCE	23		4	1	XXVIII
(26)	17	33	3	CPCE	48		3	0	IX
(27)	17	45	6	RPCE	26		6	0	XXVII
(28)	18	10	2	RPCE	48		0	2	XX
(29)	18	20	2	CPCE	47		2	0	XI
(30)	18	30	4	RPCE	48		4	0	XIV
(31)	19	30	3	RPCE	27		2	1	XXIII
(32)	20	0	3	CPCE	22		3	0	VI
(33)	20	10	2	RPCE	49		1	1	XVII
(34)	20	20	2	RPCE	47		0	2	XX

*Cell Structure and Cell Activity.*

By MARGARET PENROSE, M.A., M.R.C.S., and J. H. QUASTEL, D.Sc., F.I.C.,  
late Fellow of Trinity College, Cambridge ; Biochemical Laboratory,  
Cardiff City Mental Hospital.

(Communicated by Sir Frederick Hopkins, F.R.S.—Received July 11, 1930.)

*Introduction.*

The work of Batelli and Stern and of Warburg has made clear the importance of surface structures in tissue oxidations, and a survey of oxidation systems as a whole show that there must be a considerable grading in complexity and stability of such structures. Agents such as those which oxidise the purine bases or aldehydes, are extractable from the cell ; others have not yet been dissociated from the tissues. So many enzymes can be extracted from the cell only by means which must inevitably affect its structure that it is surprising to find that cell activity (or activity of its catalysts) is at all dependent upon cell structure. Disintegration of a cell, the breaking down of cell membranes, will certainly bring about as A. V. Hill (1928) has pointed out, a biochemical chaos and a medley of reactions. Catalysts will be brought into contact with substrates previously held remote from them. The cell end products will change in type and quantity.

But if by activity of a cell is meant the activity of its catalysts, mechanical disintegration of the cell will not, in itself, be expected to bring about any pronounced change. Muscle ground with sand exhibits a very good oxygen uptake in presence of para-phenylenediamine (Keilin, 1929). A number of dehydrogenases may be extracted from tissues by shaking or maceration in saline or alkaline phosphate solution. Succinic dehydrogenase can be obtained from muscle and lactic dehydrogenase from yeast in this way. Oxidases of compounds of the aromatic type, *e.g.*, catechol oxidase or tyrosinase appear to act independently of the cell as a whole ; so do peroxidase and the hydrolytic ferments. Failure to extract any enzyme would seem to be due to ignorance of a suitable method of elution from the tissue.

The representation of cell activity, however, as the sum total of the activities of enzymes whose sole dependence on cell structure consists in their specific or a general adsorption upon cell surfaces, is far from complete. It will not

easily account for the experimental fact that the oxygen uptake of muscle in presence of an added metabolite such as succinate is very greatly dependent upon the previous manipulation of the muscle (*e.g.*, whether it has been minced or ground with sand); nor will it account for the evidence which has accumulated from the study of bacteria. Here, on the contrary, the evidence points to a close relationship between cell structure and dehydrogenase activity (Quastel, 1926; Quastel and Wooldridge, 1927, 1928).

The inter-relation between structure and activity of a cell presents a very important aspect of cell mechanisms and cell dynamics. It is hoped in this communication to present evidence which will make clearer the nature of this relationship.

#### *Micrococcus lysodeikticus.*

This organism is particularly well suited for the investigation to be described. It has the remarkable property of exhibiting lysis in a few minutes in the presence of a dilute solution of saliva or of egg white.

Fleming (1922) showed that many secretions of the body and organ extracts had a lytic action on certain types of bacteria. He isolated an organism, given the name *M. lysodeikticus*, lysis of which was caused by tears, or saliva or egg white in very considerable dilution. Hen's egg white has a lytic effect at a concentration of 1 in 60,000,000, the yolk having no such action. The lytic agent, termed lysozyme, appears to possess considerable bactericidal powers even with organisms where lysis is not demonstrable. The lysozyme for instance has a lethal action on *B. coli* or *B. typhosus*, organisms which are resistant to lytic action.

The lytic action is rapid, taking place with, say, a 1/500 solution of egg white at neutral reaction, within 30 minutes either at 45° or 37°. The action is much more rapid at the higher temperature. With very thick suspensions of the organism lysis does not appear to be complete within this period but there is usually at least a 70 per cent. lysis. Lysozyme is thermolabile; it brings about a lysis whether the organisms be alive or dead. With the heated or autoclaved organism, however, the rate of lysis is greatly diminished. It differs very considerably from *bacteriophage*. Neither diastase nor trypsin behave in the same way. For further properties of lysozyme and its significance in the body, the papers of Fleming and Allison (1922, 1922a, 1924, 1925, 1927) and Fleming (1924) should be consulted.

The very rapid lytic action of dilute solutions of saliva or of egg white on *M. lysodeikticus* makes it possible to study the changes in the activities of its enzymes after the cell structure has been disintegrated.



*Biochemical Activity of M. lysodeikticus.*

It was necessary in the first place to study the normal activity of the intact organism. The enzymes selected for study were (1) the dehydrogenases, (2) the oxidase of para-phenylenediamine, (3) peroxidase, (4) catalase, (5) fumarase, (6) urease.

*Activation of Hydrogen Donators.*

The capacity of the cell to activate certain substrates as hydrogen donators is of great importance to its respiratory activity and indeed to its metabolism as a whole. The cell's ability to produce "active hydrogen" is one of the factors determining the nature of the chemical events which the cell can bring about. Experiments with *B. coli* and other organisms have clearly shown (Quastel, Stephenson and Whetham, 1925; Quastel and Stephenson, 1925) that one of the most important factors influencing the proliferation of these organisms is their ability to activate certain molecules as hydrogen donators. A study of the dehydrogenases (the agents responsible for the activation) of *M. lysodeikticus* was clearly essential for the problem under consideration.

The activations of the organism were determined by the methylene blue technique using Thunberg vacuum tubes. The technique is so well known that it scarcely needs a full description here. Details will be found in earlier papers (*loc. cit.*). In brief it consists in placing in a vacuum tube a suspension of the well-washed organism, a suitable quantity of the substance under investigation, a quantity of buffer solution of known  $p_H$ , and a solution of methylene blue. The tube is evacuated at a water pump and incubated in a water bath at 45°. The rate at which the dyestuff is reduced is observed. If the organism has no activating power, the substrate (which itself does not affect the dyestuff) does not accelerate the rate of reduction due to the organism alone. If an acceleration occurs this is usually due to an activation of the substrate whereby it becomes a hydrogen donator, *i.e.*, it parts with its hydrogen to the methylene blue. The technique has been used extensively for the study of dehydrogenase systems. Lately it has been used by Kendall (1929) for the investigation of the activation of a number of sugars in presence of various organisms.

The organism was prepared usually from a 30-hour growth on agar plates or slopes, the growth being suspended in normal saline and washed twice with saline. It should be used as freshly as possible; on long standing the organism acquires an increase in its own reducing power and a decrease in its capacity to activate hydrogen donators.

It was found that lactic acid, glutamic acid, succinic acid, glycerophosphoric

acid, glucose and l  vulose act as hydrogen donators in presence of the organism. The acids are presented to the organism in the form of their sodium salts. Nutrient broth (containing many amino acids, etc.) acts as a powerful source of hydrogen in presence of the organism. Table I gives an indication of the activating power of the organism.

Table I.—Reduction Times with *M. lysodeikticus*.

Each vacuum tube contained 2 c.c. M/15 phosphate buffer  $p_H$  7.4, 1 c.c. 1/5000 methylene blue solution, 1 c.c. suspension of organism, and donator, the total volume being made up to 6 c.c. with normal saline. Reductions were carried out *in vacuo* at 45°. All substances under investigation were brought to  $p_H$  7.4 by sodium hydroxide or hydrochloric acid. The following are typical results.

Donator.	Final concentration of donator.	Reduction Time.
Control (no donator)	—	2 hours
Glucose	M/60	12 minutes
L��vulose	M/60	13 ..
Succinic acid	M/15	53 ..
Glutamic acid	M/15	12 ..
Glycerophosphoric acid	M/200	17 ..
Lactic acid	M/60	6 ..
Nutrient broth	—	4 ..

#### *Oxygen Uptake by M. lysodeikticus.*

In correlation with the results obtained with methylene blue, measurements were made of the oxygen uptake of *M. lysodeikticus* by itself and in the presence of hydrogen donators. For this purpose use was made of the Barcroft differential apparatus. The right-hand flask of the apparatus received either the organism alone or the organism together with the substance under investigation. Phosphate buffer solution of  $p_H$  7.4 was always present. The left-hand flask contained either saline and buffer solution or saline and buffer solution and the same amount of organism as in the right-hand flask. The total volume in each flask was 3 c.c. The CO<sub>2</sub> absorbing tubes in each flask contained a cylindrical roll of filter paper soaked in 6 per cent. potash. All experiments were carried out in a carefully regulated water bath at 37°.

Table II gives the oxygen uptakes obtained with two typical substrates, the experiments being arranged so that there was the same amount of organism in each flask. The oxygen uptake due to the combustion of the substrate alone was thus obtained. The rate of oxygen uptake was linear.

Table II.—Oxygen Uptake by *M. lysodeikticus*.

The left-hand vessel of the Barcroft manometer contained 1 c.c. *M*/15 phosphate buffer *pH* 7.4, 1 c.c. sodium chloride solution of the same molarity as the salt under investigation in the right-hand vessel and 1 c.c. suspension of the organism. The right-hand vessel contained the same materials with the exception that the sodium chloride was replaced by sodium succinate or sodium lactate in the concentration given.

Substrate.	Final concentration of substrate.	O <sub>2</sub> uptake. (c.mm. in 90 mins.)
Succinate ....	<i>M</i> /7.5	348
Lactate .....	<i>M</i> /15	780

*The Effect of Lysis on the Activation of Hydrogen Donators.*

A stock of *M. lysodeikticus* was divided into two parts. To one was added a solution of saliva or of egg white (in saline), to the other an equal volume of saline. The two suspensions were incubated at 37° for an hour, when lysis occurred where the saliva or egg white was present. The other suspension was unchanged. The organism after lysis and the intact organism were then compared for activations of hydrogen donators by the methylene blue technique.

The effect of lysis is very striking. There is a complete elimination of the activation of the sugars and of glutamic acid. Table III gives representative results. The activity of nutrient broth is greatly diminished indicating that other amino acid dehydrogenases suffer the same fate as that for glutamic acid. Usually over 70 per cent. of the lactic dehydrogenase was destroyed. The succinic dehydrogenase results were variable but there is usually over 50 per cent. destruction. The succinic and lactic dehydrogenases were the only ones which were perceptibly active after lysis, though there was always a large diminution in activity. Nutrient broth usually gave a slight residual activity but the factor responsible for this is at present unknown.

It was very difficult to be sure in these experiments that complete lysis had occurred and that the residual reductions were not due to remaining intact cells. In a number of experiments the suspensions, after lysis, were centrifuged and the clear centrifugates examined for reducing power with lactate. They gave increased reduction times indicating the presence of either intact cells or large colloidal aggregates, capable of activating lactates in the original fluid obtained after lysis. There is little doubt, however, that after a thorough lysis there is a definite residual activity in presence of lactates and succinates.

The same effects occurred whether the lysis was due to saliva or to egg white.

Table III.

Reduction times in minutes by intact and lysed *M. lysodeikticus*. Conditions as in Table I. In each experiment, bearing the same number, the same quantity of organism was used, so that the results of these experiments can be compared quantitatively with each other. The following are typical results.

Experiment.	Donators.	Final concentration of donator.	Intact organism.	Organism lysed by 1/300 egg white.	Organism lysed by 1/100 saliva.
1	Control (saline only)	—	2 hours	Not reduced in 4 hours	Not reduced in 4 hours
1	Lactic acid .....	M/60	7	60	56
2	Control (saline only)	—	90	Not reduced in 3 hours	Not reduced in 3 hours
2	Lactic acid .....	M/60	8	27	26
2	Glucose .....	M/60	16	Not reduced in 3 hours	—
2	Nutrient broth ....	—	3	45	31
3	Control (saline only)	—	Not reduced in 3 hours	Not reduced in 3 hours	—
3	Succinic acid .....	M/15	24	73	—
3	Lactic acid .....	M/60	10	40	—
3	Levulose .....	M/60	13	Not reduced in 2 hours	—
3	Glutamic acid ...	M/60	11	Not reduced in 2 hours	—
4	Glycerophosphoric acid	M/200	17	Not reduced in 4 hours	—
4	Glucose .....	M/60	12	Not reduced in 4 hours	—
5	Glucose .....	M/60	19	—	Not reduced in 2 hours
5	Lactic acid .....	M/30	15	—	100

It is of interest that the lactic and succinic dehydrogenases should exhibit the greatest stability following lysis of the organism. The lactic enzyme is present in the products of autolysis of *B. coli* (Stephenson, 1928) and the succinic dehydrogenase in phosphate extracts of tissues. It is important to point out, however, the quantitative aspect of the phenomenon. The lysis effects a complete destruction of the sugar and amino acid enzymes and at least a 70 per cent. destruction of the more stable lactic catalyst. Possibly the remaining activity with lactate and succinate is due to intracellular material which escapes the action of the lytic agent. In view of these results it is very likely that the amounts of lactic or succinic dehydrogenases extractable from yeast or tissues represent only a fraction of the total activity of the intact cells for these substrates.

In order to ascertain whether saliva contains a thermostable factor toxic to dehydrogenases, saliva was autoclaved and added to a suspension of

*M. lysodeikticus*. No lysis occurred and an examination with the methylene blue technique showed no diminution in activating power.

*The Effects of Lysis on Aerobic Oxidations of M. lysodeikticus.*

As in the methylene blue experiments, a stock of the organism was divided into two parts; to one was added saliva or egg white and lysis allowed to occur at 37°; to the other was added an equal volume of saline and the suspension incubated at 37° for 1 hour. The oxygen uptake by the intact organism and by the organism after lysis in presence of various substrates was observed.

Table IV gives typical results.

Table IV.

Oxygen uptake by intact and lysed *M. lysodeikticus* in presence of various substrates. Conditions as in Table II. The following are typical results, which are quantitatively comparable in each experiment. All substrates were brought to pH 7.4.

Experiment.	Substrate.	O <sub>2</sub> uptake in c.mm. by intact organism.	O <sub>2</sub> uptake in c.mm. by lysed organism.	Time
1	Nutrient broth	336	57	1 hour
2	(Glucose ( <i>M</i> /30))	225	24	:
3	Lactic acid ( <i>M</i> /24)	1014	108	:
4	Succinic acid ( <i>M</i> /7.5)	414	6	:
5	Fumaric acid ( <i>M</i> /7.5)	246	7	:

The results are closely parallel to those obtained with the methylene blue technique. There is a marked diminution in the velocity of oxygen uptake, amounting in most cases to about 90 per cent.

It may be concluded from these experiments that the effect of the lytic agent on *M. lysodeikticus* is to bring about almost a complete cessation of dehydrogenase activity.

*Oxidation of p-phenylenediamine by M. lysodeikticus.*

The biological oxidation of *p*-phenylenediamine has acquired a special significance in view of the work of Warburg and Keilin. The latter has demonstrated (1929) that factors such as KCN, H<sub>2</sub>S, CO in dark or light, alcohol or acetone treatment affect *p*-phenylenediamine oxidation in much the same way as they affect the total respiration of the cell. Keilin has made it appear very probable that the *p*-phenylenediamine oxidase plays an essential part in

the normal respiratory activity of the cell and that the oxidase is identical with Warburg's "respiratory ferment."

A study of the activity of *M. lysodeikticus* was clearly of importance for the problem under consideration.

Washed *M. lysodeikticus* has a small but definite oxygen uptake in presence of *p*-phenylenediamine. The oxygen uptake was observed in the Barcroft apparatus. The *p*-phenylenediamine was added as a neutralised solution of 21 mg. in 0.3 c.c.

Curve B, fig. 1, indicates the rate of oxygen uptake by the phenylenediamine

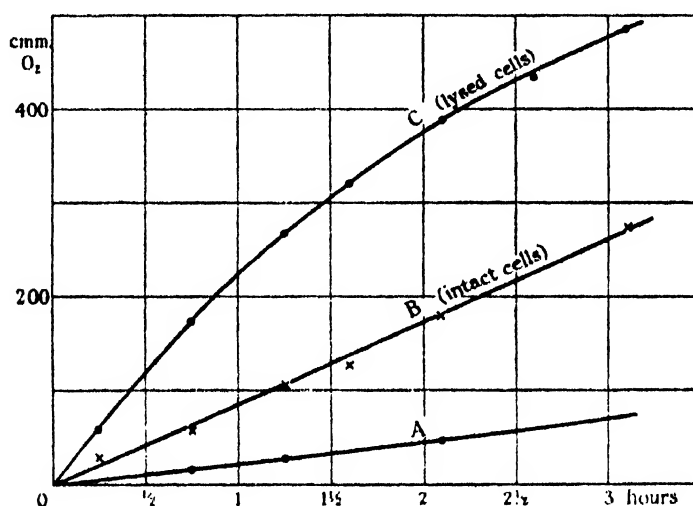


FIG. 1.—Rate of Oxygen Uptake by Intact and Lysed Cells in presence of *p*-Phenylenediamine.

in presence of the intact organism, the experiment being arranged so that there was the same amount of organism in each flask of the apparatus. Curve A shows the rate of autooxidation of the amine. The rate of oxygen uptake was linear or nearly so, and was inhibited in the presence of dilute KCN solution.

#### *The Effect of Lysis on the Oxidation of p-phenylenediamine.*

The effect of lysis was to increase the rate of oxidation of the amine. Curve C, fig. 1, illustrates this and Table V gives typical results. Control experiments showed no appreciable acceleration of the oxidation of *p*-phenylenediamine by saliva or egg white alone. The effect of boiling the organism after lysis was to remove entirely the power of accelerating the oxidation of the amine (experiment 3, Table V).

Table V.

Oxygen uptake by intact and lysed *M. Lysodeikticus* in presence of *p*-phenylenediamine. The following results represent the net uptake of oxygen in presence of the diamine (after subtracting the oxygen uptake of the organism alone). Each vessel of the Barcroft manometer contained 21 mg. of the diamine, the right hand side containing the organism.

Experiment.	O <sub>2</sub> uptake in c.mm.		
	1 hour.	2 hours.	3 hours.
Intact organism .....	51	111	162
Organism after lysis (with saliva) .....	138	354	414
Intact organism .....	—	165	—
Organism after lysis (with egg white) .....	—	228	—
Lysed organism .....	207	—	—
Lysed organism, after boiling .....	0	—	—

It should be added that the organism's own autoxidation is almost entirely eliminated by lysis.

*p*-phenylenediamine oxidation offers, therefore, a most striking contrast to the oxidation of those donators which require activation by the cell. It is already known from Keilin's work that narcotics do not affect *p*-phenylenediamine oxidation in the way that they affect dehydrogenases. The experiments which have been described make it clear that lysis of the cell does not inhibit in the least the oxidation of *p*-phenylenediamine and the obvious conclusion is that this oxidase is independent of the structure of the cell as a whole. It falls in line, in fact, with peroxidase, which, as will be shown later, is also unaffected by the lysis. The nature of the *p*-phenylenediamine oxidase is clearly different from that of the dehydrogenases in its independence of the integrity of the cell structure as a whole ; this might be expected if the oxidase belongs to that category of substances to which cytochrome is related.

From the point of view of the respiratory activity of the cell it must be concluded that activation of oxygen is not dependent on the cell structure being intact and affords therefore a fundamental difference from the activation of hydrogen which appears to be so dependent.

The increase in rate of oxidation of *p*-phenylenediamine in presence of the lysed cell is probably due to an increased facility of access of the reagent to the enzyme.

#### *The Effects of Saliva and of Egg White on the Activations of B. Coli.*

Since it is conceivable that both saliva and egg white contain factors which are toxic to dehydrogenases, quite apart from their content of lysozyme, the

effects of these substances on *B. coli* were examined, both by the methylene blue technique and by measurements of oxygen uptake in the Barcroft differential apparatus.

It was found that neither saliva nor egg white had any appreciable inhibitory action on the activations by *B. coli* on any of the donators under investigation. Neither had *B. coli*, in presence of egg white, any diminished oxygen uptake in presence of such donators as succinate or lactate.

For instance the reduction times of *B. coli* in presence of *M*/60 lactate (10 minutes), *M*/15 succinate (14 minutes), *M*/60 glucose ( $2\frac{1}{2}$  minutes), *M*/60 l  vulose ( $3\frac{1}{4}$  minutes), and nutrient broth (10 minutes) were inappreciably affected by 1 hour's preliminary incubation of the organism at 37  in presence of 1/100 saliva or 1/200 egg white. Again, the O  uptakes of untreated *B. coli* in presence of *M*/7.5 succinate and *M*/15 lactate in 1 hour at 37  were 138 c.mm. and 153 c.mm. respectively; after incubation with egg white the O  uptakes in 1 hour were 144 c.mm. and 159 c.mm. respectively.

The results are of interest in view of the fact that saliva and egg white are bactericidal to *B. coli* although they have no lytic action (Fleming). They support the conclusions of previous work that many substances which are bactericidal do not necessarily affect all the dehydrogenases; they also make clearer the connection between lytic action and destruction of dehydrogenase activity.

#### *The Effect of Saliva on Lactic Dehydrogenase.*

Since it is possible that *B. coli* is impermeable to a factor in saliva toxic to the dehydrogenases, the effect of saliva on a "soluble" dehydrogenase was examined. The most convenient enzyme was the lactic dehydrogenase which was extracted from yeast by the method suggested by Bernheim (1928).

Clearly if there is a factor in saliva which destroys over 70 per cent. of the lactic dehydrogenase of *M. lysodeikticus*, it should also have a destructive action on the lactic dehydrogenase of yeast. Experiment showed that the saliva had not the least inhibitory action. For example: The reduction time of the yeast extract with lactate alone was 28 minutes; that of the extract with 1 c.c. of 1 in 5 saliva alone was 40 minutes; and that of the extract with both the saliva and the lactate (after incubation) was 15 minutes. The theoretical time for a summation of effects was 16 minutes.

The conclusion from the experiments with *B. coli* and the extracted lactic dehydrogenase is that the destruction of dehydrogenases after lytic action is connected with the lytic action itself, i.e., that the dehydrogenases depend for their maximal activity on the cell structure as a whole being intact. It is



necessary to point out that this does not mean that internal surface structures, which are not necessarily affected by lysis, have no dehydrogenase activity. On the contrary there are relatively stable structures capable of dehydrogenase action. But when the cell as a whole is considered, it is clear from the experiments which have been described, that the total dehydrogenase activity depends greatly on the integrity of the cell structure.

#### *Peroxidase.*

The organism was subjected to lysis in the manner already described and examined for its peroxidase activity. Owing to its large content of catalase which obscured the reaction with hydrogen peroxide, the preparation was heated previous to the addition of the reagents (benzidine and hydrogen peroxide). A typical peroxidase reaction ensued which was not appreciably different from that given by the intact organism.

#### *Catalase.*

The effect of lysis on *M. lysodeikticus* is to bring about a considerable increase in activity towards hydrogen peroxide.

The experiment is performed in the apparatus usually used for the estimation of urea by the hypobromite method. The apparatus consists simply of a burette, the upper end of which is attached to a wide mouthed bottle containing a short tube which rests inclined to the base of the bottle at the commencement of the experiment. In the wide mouthed bottle are placed 1 c.c. suspension of the organism, 2 c.c. phosphate buffer solution  $p_H$  7.4 and 1 c.c. 20 vol. neutral  $H_2O_2$ . In the short tube is placed 1 c.c. of 1/50 saliva or egg white. The bottle is connected to the burette and the rate at which oxygen is evolved measured, at a known temperature and at atmospheric pressure. At a given time the short tube in the bottle is inverted and the effects of the addition of the saliva or egg white to the organism noted. Fig. 2 represents the course of the reaction, the egg white being added at zero time (curve B). Curve A shows the course of action of the intact organism.

In a few seconds after the addition of saliva or egg white to the organism there is a marked, almost violent, increase in the rate of evolution of oxygen. It is necessary to use very little organism in the experiment in order to follow the rate. But at the point at which the sudden increase in activity occurs there is no apparent lysis. The lytic agent appears to have a preliminary effect, prior to lysis, resulting in a much more rapid decomposition of hydrogen

peroxide. This phenomenon does not occur with *B. coli*. Here the rate of evolution of oxygen is unchanged by the addition of saliva or egg white.

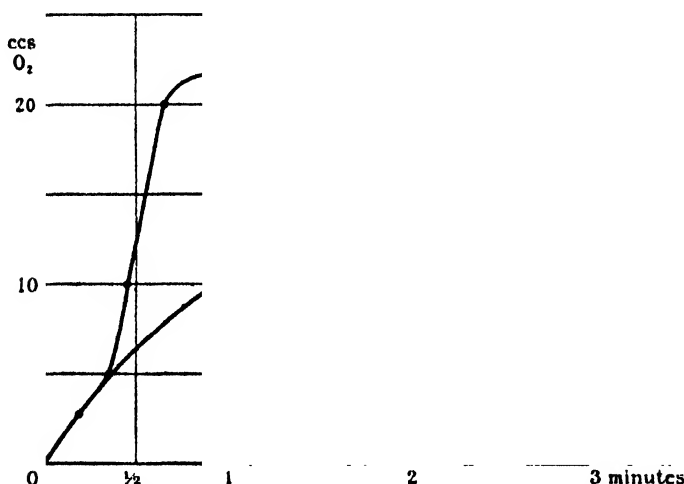


FIG. 2.—Effect of Egg White on Decomposition of  $H_2O_2$  by *M. lysodeikticus*.

The simplest explanation of the phenomenon is that the lytic agent effects very quickly some change in the cell surface, whereby the cell becomes far more permeable to the hydrogen peroxide; as the change goes on ultimate lysis occurs. The phenomenon is worthy of close attention, for it is readily amenable to strictly quantitative treatment.

#### *Fumarase.*

This enzyme which converts fumaric acid into *l*-malic acid has an extremely wide biological distribution and was first described in the bacteria in the case of *B. coli* (Quastel and Whetham, 1924). *M. lysodeikticus* contains the enzyme and the effect of lysis on the activity of the organism towards fumaric acid was examined.

The method consisted in adding to a suspension of the organism a quantity of phosphate buffer solution  $p_H$  7.4 and a solution of sodium fumarate to make a final concentration 0.08 *M*. The final concentration of phosphate was usually 0.04 *M*. Incubation was carried out either at 37° or at 45°. The production of *l*-malic acid was followed polarimetrically. To 5 c.c. of the solution were added 1 c.c. glacial acetic acid and 10 c.c. 14.2 per cent. ammonium molybdate solution. The solution was centrifuged and the centrifugate examined in the polarimeter.

Precisely the same phenomenon was found with fumarase as with catalase. The intact organism showed very little *l*-malate production within the first 3 hours' incubation at 45° but the organism after lysis in the same period converted the fumarate into the equilibrium concentration of *l*-malate (75 per cent. conversion). The lytic agent itself had no fumarase activity.

For example, whereas with the organism intact, the solution after incubation for 3 hours at 45° in presence of 0.08 M fumarate, gave no appreciable rotation, that with the organism after lysis with 1/200 egg white gave (under the same conditions) a large positive rotation indicating the production of 0.06 M *l*-malate.

After 24 hours' incubation at 37° the intact organism converted the fumarate into the equilibrium concentration of *l*-malate.

As with hydrogen peroxide, so it appears with fumarate, the lytic agent produces an enhancement of activity, probably due to an easier access of the fumarate to its enzyme.

#### *Urease.*

Results with urease were much the same as with fumarate and hydrogen peroxide. The effect of lysis was to increase the rate at which urea was converted into ammonia.

For experiment a suspension of the intact organism, together with phosphate buffer at  $p_H$  7.4 and a solution of urea giving a final concentration of 0.8 per cent. was compared with the organism after lysis under like conditions. Incubation was carried out for 1 or 2 hours at 45°. Free ammonia was estimated in the usual way by aeration from a solution made alkaline with potassium carbonate, into standard acid. Controls were carried out with the lytic agents to ensure the absence from these of any urease.

In a typical experiment the intact organism yielded 12.27 c.c. N/10  $NH_3$  in 90 minutes at 45°, whereas the same amount of organism after lysis produced 21.03 c.c. N/10  $NH_3$  under the same conditions.

Again it is clear that lysis effects a change bringing about an increased rate of action.

#### *Conclusions and Summary.*

The results described are for convenience summarised in Table VI.

The effects of lysis of *M. lysodeikticus* are, on the one hand, to bring about a diminution or elimination of the activation of hydrogen donors, and on the other hand to produce an increased activity of the oxidase of *p*-phenylenediamine and of the enzymes catalase, fumarase and urease. The increase in activity is probably due to an increased facility of access of the reagents to

Table VI.

Enzyme.	Effect of lysis.
Catalase .....	Increased action.
Fumarase .....	Increased action.
Urease .....	Increased action.
Peroxidase .....	No apparent change.
Oxidase of <i>p</i> -phenylenediamine .....	Increased action.
Dehydrogenase of—	
Glucose or fructose .....	Activity completely destroyed.
Lactic acid .....	Activity diminished by 70 per cent.
Succinic acid .....	Activity diminished by 50 per cent.
Glutamic acid .....	Activity completely destroyed.
Glycerophosphoric acid .....	Activity completely destroyed.
Aerobic oxidation of hydrogen donors .....	Activity largely eliminated (reduction about 90 per cent.).

the enzymes. Peroxidase appears to be unaffected by the lysis. The conclusion would be that maximal dehydrogenase activity, which is responsible for the source of active hydrogen in the cell, is inherently connected with the integrity of cell structure, whilst the activation of oxygen is dependent upon a factor whose activity, like that of catalase, peroxidase, fumarase or urease, is independent of cell integrity.

The authors have pleasure in expressing their very great appreciation of the assistance given them by Mr. A. H. M. Wheatley, B.Sc. Mr. Wheatley is in receipt of a grant from the Medical Research Council. To this body, too, the authors are grateful for a grant in aid of equipment.

#### REFERENCES.

- Bernheim (1928). 'Biochem. J.,' vol. 22, p. 1178.  
 Fleming (1924). 'Lancet,' p. 217.  
 Fleming and Allison (1922). 'Proc. Roy. Soc.,' B, vol. 94, p. 142.  
 Fleming and Allison (1922). 'J. Exp. Path.,' vol. 3, p. 252.  
 Fleming and Allison (1924). 'Lancet,' p. 1303.  
 Fleming and Allison (1925). 'J. Exp. Path.,' vol. 6, p. 87.  
 Fleming and Allison (1927). 'J. Exp. Path.,' vol. 8, p. 214.  
 Hill, A. V. (1928). 'Proc. Roy. Soc.,' B, vol. 103, p. 138.  
 Keilin (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 206.  
 Kendall (1929). 'J. Infect. Dis.,' vol. 44, p. 282.  
 Quastel (1926). 'Biochem. J.,' vol. 20, p. 166.  
 Quastel and Stephenson (1925). 'Biochem. J.,' vol. 19, p. 660.  
 Quastel, Stephenson and Whetham (1925). 'Biochem. J.,' vol. 19, p. 304.  
 Quastel and Whetham (1924). 'Biochem. J.,' vol. 18, p. 519.  
 Quastel and Wooldridge (1927). 'Biochem. J.,' vol. 21, pp. 148, 1224.  
 Quastel and Wooldridge (1928). 'Biochem. J.,' vol. 22, p. 689.  
 Stephenson (1928). 'Biochem. J.,' vol. 22, p. 605.

*The Frozen State in Mammalian Muscle.*

By T. MORAN (Food Investigation Board of the Department of Scientific and Industrial Research and Low Temperature Research Station, Cambridge).

(Communicated by Sir William Hardy, F.R.S.—Received July 18, 1930.)

In common with the majority of colloidal systems, mammalian muscle when frozen undergoes serious structural change, part of the water previously held within the muscle fibres being expelled on thawing as a synaeretic fluid. The trend of recent work on the freezing of tissues has been to minimise the factor of cold and to regard the equilibrium effects of freezing as being produced by a certain measure of desiccation. In the case of living muscle an exact correlation between freezing and drying has been definitely established (Moran, 1929; Smith, 1929; Smith and Moran, 1930). One of the difficulties, however, in extending the concept to mammalian muscle, post-mortem, is the lack of data as to the amounts of water actually present as ice at different freezing temperatures. The present paper describes the results of an attempt to supply this need for three examples of mammalian muscle, namely, beef, mutton and pork.

*The Dilatometric Behaviour of Frozen Muscle.*

There are several methods of determining the extent of the ice separation in frozen muscle, but probably the two most direct are the calorimetric and dilatometric methods. The latter has the advantage of extreme simplicity and it was this which was used in the present work.

The dilatometer was of the ordinary closed bulb and capillary stem type; the dimensions of the bulb being those of a cylinder approximately 30 cm. long and 1.7 cm. internal diameter; the capillary averaged 90 cm. in length and 0.014 sq. cm. in cross section. The capillary was calibrated at 0° C. by determining the weight of a measured column of mercury. The method was accurate to 1 in 300 and no difference could be detected between this and the calibration at -20° C. Ordinary medicinal liquid paraffin was chosen to mark the changes in volume as this liquid is the one least likely to have any action on the muscle. Perhaps the most direct piece of evidence in support of this is the fact that living amphibian muscle will retain its irritability in

oxygenated liquid paraffin at  $0^{\circ}\text{C}$ . for several days, until in fact the muscle begins to putrefy. The weight of muscle\* used in each experiment was adjusted to approximately 10 gm. and then accurately weighed; it was introduced into the dilatometer in the form of small pieces which were virtually suspended in the liquid paraffin. This reduced the possibility of slight deformation of the walls of the dilatometer during freezing. The volume of the system, as indicated by the position of the paraffin in the capillary, was determined accurately at several temperatures between  $0^{\circ}\text{C}$ . and  $-20^{\circ}\text{C}$ . At each temperature the dilatometer was stored for several days to ensure that equilibrium was attained. The average time taken over the complete freezing-thawing cycle was 50 days. Fig. 1 represents the type of volume-temperature curve obtained.

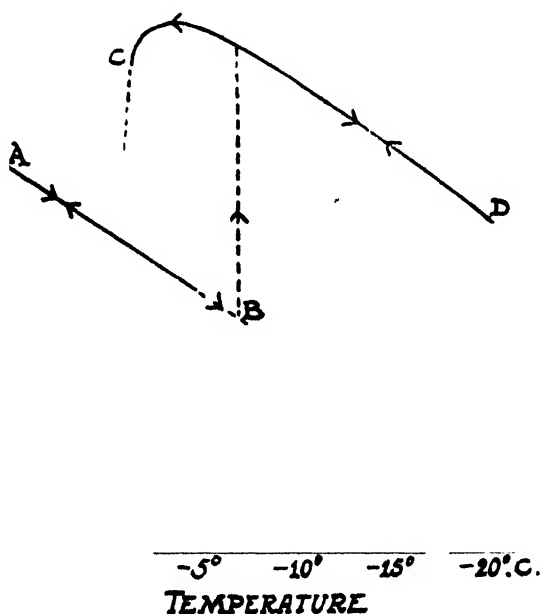


FIG. 1.

### *The Changes in Volume.*

The increase in volume due to ice formation at the different temperatures was calculated from the difference in the readings on CD and AB (or AB

\* The muscle when introduced into the dilatometer had been dead between 24 and 48 hours. The cooling of the dilatometer was made as rapid as possible, but even so the average time that elapsed between the death of the muscle and its freezing was approximately 5 days. For 3 to 4 days out of this period the temperature of the muscle was in the range  $0^{\circ}$  to  $-5^{\circ}\text{C}$ .

produced) together with a small correction at  $-20^{\circ}\text{C.}$  to allow for the fact that contraction of the liquid paraffin ceased to be linear below  $-10^{\circ}\text{C.}$  In view of the fact that the changes in the specific volume of supercooled water are not exactly linear with temperature, extrapolation of AB below  $-5^{\circ}\text{C.}$  is not strictly accurate. It can be shown, however, that the maximum possible error at  $-10^{\circ}\text{C.}$  in the calculated amount of water frozen out is 0.65 per cent.

Experiments were carried out in duplicate on lean muscle from beef topside (*biceps femoris*), and on the loin muscles of mutton and pork. The total water in each muscle was determined by drying a similar sample at  $103^{\circ}\text{C.}$  for 1 week. The results when plotted, as the increases in volume at each temperature per 1 gm. of water in the muscle were characteristic of one curve and not three separate curves. The changes in volume taken from the smoothed curve are shown in Table I. The maximum experimental deviation from these values did not exceed 2 per cent.

#### *The Amount of Ice Formed.*

To calculate the amounts of water frozen out at the different temperatures from the observed changes in volume it is necessary to know the specific volumes of ice and supercooled water at each temperature. The data used were the average of the more concordant values given in Landolt-Bornstein *Physikalisch-Chemische Tabellen* (1923), namely:—

Density of ice at  $0^{\circ}\text{C.} = 0.9168$  (p. 311).

Coefficient of contraction of ice between  $0^{\circ}$  and  $-20^{\circ}\text{C.} = 0.000156$  (p. 1221).

Specific volume of supercooled water = mean of the values found by Pierre, etc., and Mohler (p. 73).

Unfortunately the specific volume of supercooled water at  $-20^{\circ}\text{C.}$  is not available nor is it satisfactory to fix an *absolute* value by extrapolation. The value taken was that obtained by a linear extrapolation of the values at  $0^{\circ}$  and  $-10^{\circ}\text{C.}$  Whilst this value is not strictly accurate the error introduced will be almost entirely counterbalanced by the fact that the increase in volume of the muscle due to freezing at  $-20^{\circ}\text{C.}$  was obtained by a linear extrapolation of AB (fig. 1).

Owing to the complex nature of the salts in muscle it is impossible to allow for the slight changes in volume due to electrostriction effects when the aqueous phase of the muscle is concentrated by freezing. If,

however, as an approximation, the salt solution in muscle be regarded as the equivalent of a solution containing 0.94 gm. of NaCl in 100 gm. of water (Hill and Kupalov, 1930), and the contraction due to changes in the concentration of the sodium chloride be taken as 10 c.c. per grammole (Lamb and Lee, 1913), then the possible error at  $-20^{\circ}\text{C}$ . in the calculated amount of water frozen out is very approximately — 2 per cent.

A further possibility of slight error lies in the fact that it is impracticable to remove all the air from muscle by evacuation. If one-tenth of the total air remains in solution and, on freezing, separates out in the form of bubbles inside the muscle, the values for the percentages of water frozen out at the lower temperatures will be slightly too great. The maximum error at  $-20^{\circ}\text{C}$ . is unlikely to exceed 2.5 per cent.; at temperatures above  $-8^{\circ}\text{C}$ . the error will be negligible.

The amounts of ice formed in the muscle at the different temperatures are given in Table I.

Table I.

Equilibrium temperature.	Increase in volume per 1 gm. of water in the muscle.	Specific volume of supercooled water.	Specific volume of ice.	Percentage of total water in the muscle present as ice.
$^{\circ}\text{C}$ .	c.c.	c.c.		
— 1.5	0.0320	1.00027	1.09049	35.5
— 2.0	0.0500	1.00032	1.09041	55.5
— 3.0	0.0627	1.00043	1.09024	69.8
— 5.0	0.0727	1.00076	1.08990	81.6
— 7.0	0.0784	1.00118	1.08956	88.7
— 10.0	0.0819	1.00196	1.08905	94.0
— 20.0	0.0821	1.00379	1.08735	98.2

### Discussion.

The amounts of water present as ice in muscle at different temperatures are shown graphically in fig. 2.

The general trend of the values and the nearness to 100 per cent. of the percentage of water frozen out at  $-20^{\circ}\text{C}$ . suggests that there is very little, if any, "bound water" in the muscle. This is in agreement with the recent work of A. V. Hill (1930).

The possibility noted by Fisher (1924) in his analysis of dilatometric technique, that part of the water in muscle, as in soils, may be present in capillary spaces is also unlikely for the same reason. As he points out the specific volume of



this type of water would be greater than that of ordinary free water, in which case the volume changes recorded in Table I would at any one temperature

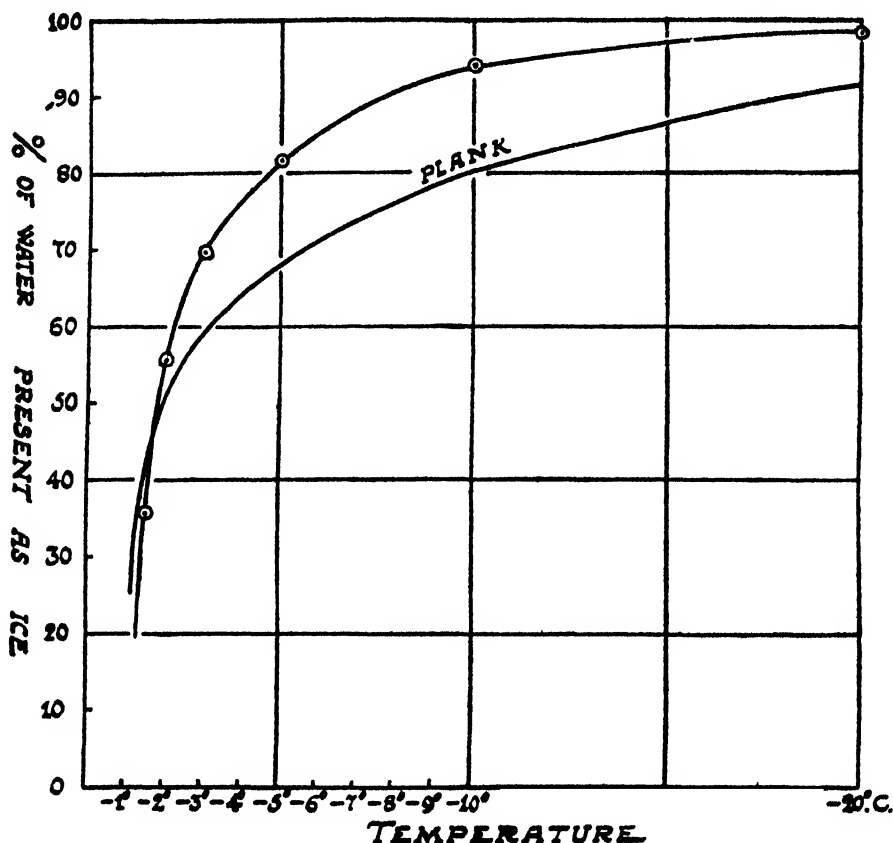


FIG. 2.

correspond to the freezing of an even greater percentage of water than is given in the table. Furthermore, it is unlikely that the eutectic point of the muscle salts is higher than  $-20^{\circ}\text{C}$ . (Smith and Moran, 1930).

The only other work on the subject is that of Plank (1925) who used a calorimetric method to determine the amounts of ice present in ox muscle at different temperatures. The objection to this method is the lack of exact data on the specific heats and latent heats of fusion of the components of muscle below the freezing point. Plank's results are also shown in fig. 2. There is a marked divergence between his results and those obtained in the present work. Hill (1930) also refers to the work of Rubner who, using the calorimetric method, at one temperature only, viz.,  $-20^{\circ}\text{C}$ ., found that 75

per cent. of the water in beef muscle is frozen. This figure is very different from that found by Plank at this temperature, namely, 91 per cent.

The dilatometric method is probably of limited value in following the molecular changes which take place when muscle is frozen since at any one temperature the volume on thawing—measurable to the extent of 0.0014 c.c. per gram of muscle—is identical with the volume before freezing. Further the volume-temperature curve obtained for a second freezing-thawing cycle is identical with the first.

### *Summary.*

Measurements have been made of the amounts of ice present in mammalian muscle when frozen to equilibrium at different temperatures in the range  $-1.5^{\circ}$  to  $-20^{\circ}$  C.

The results indicate that there is very little "bound water" (not more than 6 per cent.) in the muscle.

### REFERENCES.

- Fisher, E. A. (1924). 'J. Phys. Chem.,' vol. 28, p. 360.  
Hill, A. V. (1930). 'Proc. Roy. Soc.,' B, vol. 106, p. 477.  
Hill, A. V., and Kupalov, P. S. (1930). 'Proc. Roy. Soc.,' B, vol. 106, p. 445.  
Lamb, A. B., and Lee, R. E. (1913). 'J. Amer. Chem. Soc.,' vol. 35, p. 1666.  
Moran, T. (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 177.  
Plank, R. (1925). 'Z. ges. Kalteind.,' vol. 32, p. 141.  
Smith, E. C. (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 198.  
Smith, E. C., and Moran, T. (1930). 'Proc. Roy. Soc.,' B, vol. 106, p. 122.

*The Functions of the Corpus Luteum.—IV. The Relation of  
Œstrin to the Luteal Phase of the Œstrous Cycle.*

By A. S. PARKES, Beit Memorial Research Fellow, University College, London.

(Communicated by C. L. EVANS, F.R.S.—Received July 19, 1930.)

[PLATES 14–16.]

*I. Introduction.*

The classic work of Fraenkel, of Ancel and Bouin, and of Marshall (see Marshall, 1922, and Parkes, 1929, for discussion and references) showed conclusively that the post-ovulation changes in the accessory reproductive organs are controlled by the development of the corpus luteum. This correlation has since been worked out in considerable detail by various investigators, including Evans, and Hammond, and Courier (see Parkes, 1929, for references), and very recently Corner and his co-workers (1929) have prepared from corpora lutea extracts capable of causing the uterine changes characteristic of the luteal phase.

On the other hand, the recent intensive work on the œstrus-producing hormone, together with the delay in preparing active corpus luteum extracts, has resulted in many attempts to show that œstrin is responsible for the post-ovulation changes as well as for those typical of œstrus. In particular, the effects of œstrin on the mammary gland have been put forward as evidence that this hormone is responsible for their complete development as found at the end of pregnancy (see Parkes, 1929, for references and discussion). This supposition received superficial support from the discovery that large amounts of œstrin occur in the blood and urine during human pregnancy. Whatever the meaning of this fact may be, however, the view that œstrin is responsible directly for the changes of the luteal phase is untenable (Marshall, 1922) and has largely arisen from inadequate acquaintance with the cycle of the normal animal.

The experimental production of abortion by the administration of œstrin during pregnancy suggested that the œstrus-producing hormone was antagonistic to the action of that of the corpus luteum, but it now seems more probable that the two are to some extent complementary. Courier (1928) was able

to terminate pregnancy in its early stages in the rabbit by injection of œstrin, without affecting the glandular proliferation of the endometrium in a sterile section of the uterus, and it has also been shown by Weichert (1928) and by Corner and his co-workers (Allen, 1930) that the immature or ovariectomised animal must initially be brought under the influence of œstrin before the luteal hormone can act upon the uterus.

If œstrin could by itself produce the changes characteristic of pregnancy or pseudo-pregnancy, then continued injection of the ovariectomised animal should lead to the occurrence of a luteal phase. Animals such as the ferret and the rabbit which remain in œstrus indefinitely in the absence of copulation, without showing any further development, make it fairly evident that prolonged action of œstrin only results in prolonged œstrus, and the experimental results of prolonged injection do not warrant any other conclusion (Courrier and Masse, 1928). The development of the uterus and mammary gland following prolonged injection of œstrin, though in some instances rather more than found during normal œstrus, is trifling compared with that typical of the luteal phase. At the same time the amounts of œstrin administered have been comparatively small, and it seemed desirable to investigate the effects of continued injection of comparatively large amounts of the œstrus-producing hormone.

The work reported in the present paper was concerned with : (a) the effects of prolonged injection of large amounts of œstrin, and (b) the effect of œstrin administered during pseudo-pregnancy. The work was carried out on rabbits and ferrets, and some additional observations are recorded on the normal cyclic changes in the latter species.

## II. *Material and Technique.*

*Operative Technique.*—Ovariectomy of the ferrets and rabbits was carried out as previously described for mice—two lateral dorsal incisions being made. A much greater degree of asepsis is, however, required for the larger animals. Vasectomy of the male rabbit to allow of sterile mating was carried out as described by Hammond and Marshall (1925).

*œstrin.*—The œstrin used was prepared by Mr. G. F. Marrian by methods described by him (1929, 1930), most of the preparations containing 100 m.u. per cubic centimetre. All injections were made subcutaneously. Owing to the increased efficacy of water-soluble œstrin when given as a series of injections, the daily dosage was given in two halves night and morning.

*Histological Technique.*—All material for histological examination was

fixed in Bouin's fluid and stained in Ehrlich's hæmatoxylin and eosin. Rabbit mammary glands were stripped from the skin and prepared whole as described by Hammond (*loc. cit.*). The vaginal smears of the ferret were stained with Leischman's compound, or, where many cornified cells were involved, with saffronin.

### III. *Experiments on Rabbits.*

Two series of experiments have been carried out on rabbits: (a) prolonged injection of the immature or ovariectomised rabbit with œstrin, and (b) injection of the pseudo-pregnant rabbit with œstrin. The details and results are summarised in Table I. From this table and from the illustrations in Plates 14 and 15 the following conclusions may be drawn:—

- (a) Small amounts of œstrin (up to 25 m.u. per day) cause no appreciable change in the mammary glands of the intact or recently ovariectomised immature rabbit (POR 5–8). In the four rabbits used, however, a normal adult œstrous condition was produced in the uteri (Plate 15, fig. 6), but no signs of pseudo-pregnancy occurred. The uteri of the intact animals were similar to those of the ovariectomised, a result which does not accord with the recent report (Meyer, 1930) that œstrin injection decreases the amount of ovary-stimulating hormone in the anterior pituitary body. In view of this report, however, it is clearly desirable that work on the effects of œstrin on the uterus and mammary glands should be carried out on ovariectomised and not merely on immature animals.
- (b) Large amounts of œstrin, up to 1200 m.u. per day, given to adults ovariectomised for some months (POR 12 and 15), cause development of the mammary glands in excess of that found at normal œstrus; but this growth largely relates to the ducts and is in no way comparable to the ultimate development found during pregnancy (Plate 14, fig. 2). The uteri of animals receiving these large amounts of œstrin showed no signs of pseudo-pregnant change (Plate 15, fig. 4). In fact, the growth produced, barely equal to that of the normal œstrus condition, was definitely less than that found in POR 5 and 6 receiving much less œstrin. This discrepancy is presumably due either to the immaturity of POR 5 and 6, or else to the greater atrophy of the uteri of POR 12 and 15, owing to the greater length of time after ovariectomy. Injection of large amounts of œstrin to the castrated male causes only a slight development of the mammary glands (Plate 14, fig. 3).

Table I.—Effect of Estrin on Immature, Ovariectomised and Pseudo-pregnant Rabbits.

No. of animal.	Condition.	M.u. of estrin per day.	Days injected.	Total units.	Time of killing. Days after first injection.	Uterus.	Mammary glands.	Notes.
POR 7	Immature	12	9	108	10	Estrus	Normal immature	See Plate 15, fig. 6.
POR 8	"	12	9	108	10	"	"	
POR 5	Ovariectomised (12 days) immature	25	9	225	10	"	"	
POR 6	"	25	9	225	10	"	"	
POR 12	Ovariectomised adult (25 weeks)	300	29	8700	30	Scarcely oestrous growth	Extensive growth of ducts	{ Copulated readily on several occasions. See Plate 14, fig. 2, and Plate 15, figs. 1 and 2.
POR 15	Ovariectomised adult (14 weeks)	1200	20	24000	21	"	"	
POR 1	pseudo-pregnant 1 day	400	8	3200	10	Normal pseudo-pregnant	Normal pseudo-pregnant	
POR 2	"	400	6	2400	8	"	"	
POR 3	"	400	6	2400	8	"	"	
POR 9	2 days pseudo-pregnant	1200	11	13200	12	"	"	See Plate 14, fig. 4.
POR 10	3 days pseudo-pregnant	1200	5	6000	6	"	"	
POR 17	Castrated male	1200	20	24000	21	—	Slight growth	See Plate 14, fig. 3.

- (c) The administration of large amounts of œstrin during pseudo-pregnancy has no effect on the usual uterine and mammary changes occurring during this phase (Plate 14, fig. 4). It may be concluded, therefore, that œstrin is not antagonistic to the action of the luteal hormones.

#### IV. *Experiments on Ferrets.*

*Vaginal Smear of the Ferret.*—The external swelling of the vulva which characterises œstrus in the ferret, described with other features of the œstrous cycle by Marshall (1904) and Hammond and Marshall (1930), is in practice a perfectly adequate diagnostic sign of heat, but there is no similar external distinction between anœstrus, pseudo-pregnancy, pregnancy or lactation. It was therefore thought desirable to examine the cyclic changes, if any, occurring in the vaginal contents, as a possible method of distinguishing between the various phases of the cycle.

As a result of examining the vaginal contents, it was found that during the condition of œstrus very extensive growth and subsequent cornification of the vaginal epithelium takes place, with the consequent appearance of the characteristic cornified cells in a smear made of the vaginal contents. This cornification is similar, of course, to that found in many other mammals at œstrus.

During anœstrus it is difficult to obtain a smear of the vaginal contents owing to the atrophic state of the vagina and vulva. Nucleated epithelial cells and leucocytes are, however, present in small numbers (Plate 16, fig. 1). As the vulva begins to swell at the beginning of the breeding season the leucocytes gradually decrease, and cornified cells appear. In about a fortnight, by the time the vulva is fully swollen, cornified cells only are found (Plate 16, fig. 2). The vaginal contents consist purely of cornified cells during the whole time that œstrus lasts, except for the occasional presence of a few odd leucocytes. Copulation not resulting in ovulation has no effect on the smear. After ovulation, leucocytes and nucleated epithelial cells reappear in the smear in 2 to 3 days, *i.e.*, 4 to 5 days after copulation, and cornified cells are no longer present (Plate 16, fig. 3). If pregnancy does not follow ovulation, the smear of nucleated epithelial cells and leucocytes persists for some 7 weeks, corresponding to the duration of pseudo-pregnancy. If the breeding season is not at an end, cornification of the vagina, coincident with the return of œstrus, occurs within the next week (Plate 16, fig. 4). If copulation is fertile, the vaginal contents during pregnancy are similar to those during pseudo-pregnancy, except that a certain amount of mucus is present. Similar conditions are found during lactation, when no œstrus and no vaginal cornification is found (Plate 16, fig. 5).

Table II.—Effect of Estrin on Anestrous and Ovariectomised Ferrets.

No. of animal.	Condition.	M.u. of estrin per day.	Days injected.	Time of autopsy after first injection (days).	Ovary.	Uterus.	Vaginal smear.	Vulva.	Mammary glands.
Ferret 1	Anestrous	25	10	Not killed	—	—	Not examined	No effect	—
" 2	"	25	10	11	No effect	Slight growth	"	Very slight effect	No effect
" 3	"	50	20	44	"	"	Cornified towards end of injection	Some effect	"
" 4	"	50	20	21	"	Some growth	"	"	"
" 5	Ovariectomised 45 weeks	600 (11 days) 1200 (15 days)	26	27	—	Complete estrus	Cornified	Complete estrus growth	"
" 6	Ovariectomised 21 weeks	No injection	—	—	—	Atrophic	Anestrous	Anestrous	—



*Effect of Œstrin on Anæstrous and Ovariectomised Ferrets.*

Experiments on six ferrets are summarised in Table II. From these animals the following conclusions may be drawn :—

- (a) Small amounts of œstrin (up to 50 units) injected daily into the anæstrous ferret cause slight development of the vulva and uterus, but only a fraction of the normal œstrous growth is produced by these amounts, although cornification of the vaginal epithelium occurs.
- (b) Double ovariectomy of the adult ferret leads to atrophy of the uterus and vagina (Plate 15, fig. 3). Prolonged administration of large amounts of œstrin in this condition leads to the appearance of normal œstrous changes in the uterus (Plate 15, fig. 4), vulva and vaginal epithelium (Plate 16, fig. 8). Although injection was carried on for 26 days, no changes whatever characteristic of the luteal phase appeared.

The results on ferrets are thus in keeping with those on rabbits ; prolonged action of œstrin has no effect other than the induction of prolonged œstrous symptoms.

My thanks are again due to Prof. C. Lovatt Evans, F.R.S., and Prof. J. P. Hill, F.R.S., for facilitation of the work described. To Mr. G. F. Marrian I am very greatly indebted for generously supplying the œstrin required.

The expenses of the research were defrayed from grants from the Medical Research Council.

*VI. Summary.*

1. The prolonged administration of large amounts of œstrin (up to 25,000 mouse units in 3 weeks) to immature, ovariectomised or anæstrous rabbits and ferrets leads to the typical œstrous condition of the uterus, but none of the changes typical of pseudo-pregnancy, *i.e.*, of the luteal phase, appear.

2. In the mammary glands an accentuation of the œstrous growth of the ducts leads to a superficial resemblance to the condition found in early pseudo-pregnancy, but the extensive growth of alveoli characteristic of the luteal phase does not occur.

3. In the ferret, cornification of the vaginal epithelium, in addition to the vulval swelling, is found during œstrus, but both disappear during the luteal phase. Prolonged œstrin injection causes the prolonged appearance of both of these symptoms of œstrus.

4. The pseudo-pregnant changes in the accessory organs are, therefore, not produced by even the prolonged action of œstrin alone, and this hormone is not, therefore, the causative factor in the changes of the luteal phase.

5. On the other hand, œstrin is not antagonistic to the uterine and mammary changes of œstrus; in fact, as shown by various workers (Allen, 1930; Weichert, 1928), it is necessary to prepare the uterus for the action of the hormone of the corpus luteum.

DESCRIPTION OF PLATES 14-16.

PLATE 14.

- FIG. 1.—Photograph of mammary gland of rabbit during first œstrus, showing degree of growth typical of œstrus. Natural size.
- FIG. 2.—Photograph of mammary gland of POR 15, ovariectomised virgin adult receiving 24,000 m.u. of œstrin in 20 days. Some growth, largely of the ducts, but no approach to the full development as found during pregnancy. Natural size.
- FIG. 3.—Photograph of mammary gland of adult castrated male rabbit receiving 24,000 m.u. of œstrin in 20 days. Growth equal to the pre-pubertal female. Natural size.
- FIG. 4.—Photograph of mammary gland of rabbit receiving 13,200 m.u. of œstrin in 11 days during pseudo-pregnancy. No inhibition of pseudo-pregnant growth.

PLATE 15.

- FIG. 1.—Fold of uterine endometrium of rabbit towards the end of pseudo-pregnancy, showing development of the glands.  $\times 33$ .
- FIG. 2.—Fold of the uterine endometrium of POR 15, ovariectomised adult rabbit receiving 24,000 m.u. of œstrin. No sign of pseudo-pregnant development.  $\times 30$ .
- FIG. 3.—Uterus of adult ferret, 21 weeks after double ovariectomy, showing atrophic condition.  $\times 30$ .
- FIG. 4.—Uterus of adult ferret receiving 24,000 m.u. of œstrin over 26 days, 45 weeks after ovariectomy. Normal œstrous condition (note the large basal glands), no sign of pseudo-pregnancy.  $\times 30$ .
- FIG. 5.—Fold of uterine endometrium of normal œstrous rabbit, showing condition of glands.  $\times 30$ .
- FIG. 6.—Fold of uterine endometrium of POR 8 showing œstrous condition.  $\times 30$ .

PLATE 16.

- FIG. 1.—Vaginal smear of normal anœstrous ferret. Leucocytes and nucleated epithelial cells present.  $\times 100$ .
- FIG. 2.—Vaginal smear of normal œstrous ferret at time of copulation. Cornified epithelial cells present.  $\times 100$ .
- FIG. 3.—Vaginal smear of pseudo-pregnant ferret, 5 days after sterile copulation. Large nucleated epithelial cells and leucocytes present.  $\times 100$ .
- FIG. 4.—Vaginal smear of pregnant ferret. Leucocytes, some epithelial cells and mucus present.  $\times 100$ .
- FIG. 5.—Vaginal smear during lactation. Leucocytes and epithelial cells present.  $\times 100$ .
- FIG. 6.—Vaginal smear of anœstrous ferret injected with anterior pituitary preparations showing the cornified cells typical of œstrus.  $\times 100$ .

FIG. 7.—Vaginal smear of ferret (F5) after double ovariectomy. Smear similar to normal anoestrous type.  $\times 100$ .

FIG. 8.—Vaginal smear of ovariectomised ferret (F5) injected with oestrin, showing causation of oestrous cornification.  $\times 100$ .

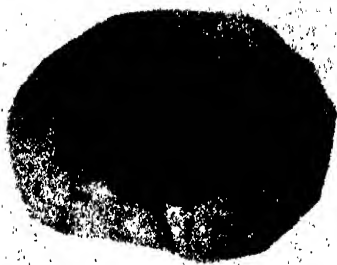
FIG. 9.—Vaginal epithelium of ovariectomised ferret (F5) injected with oestrin, showing cornified epithelium.  $\times 130$ .

#### REFERENCES.

- Allen (1930). 'Amer. J. Phys.,' vol. 92, p. 612.  
 Corner and Allen (1929). 'Amer. J. Phys.,' vol. 88, pp. 326, 340.  
 Courrier (1928). 'C. R. Soc. Biol.,' vol. 99, p. 224.  
 Courrier and Masse (1928). 'C. R. Soc. Biol.,' vol. 99, p. 265.  
 Hammond and Marshall (1925). "Reproduction in the Rabbit," Edinburgh.  
 Hammond and Marshall (1930). 'Proc. Roy. Soc.,' B, vol. 105, p. 607.  
 Marrian (1929). 'Biochem. J.,' vol. 23, pp. 1090, 1223.  
 Marrian (1930). 'Biochem. J.,' vol. 24, p. 435.  
 Marshall (1904). 'Quart. J. Mier. Sci.,' vol. 48.  
 Marshall (1922). "The Physiology of Reproduction," London.  
 Meyer (1930). 'Proc. Soc. Exp. Biol. Med.,' vol. 27, p. 702.  
 Parkes (1929). "The Internal Secretions of the Ovary," London.  
 Weichert (1928). 'Proc. Soc. Exp. Biol. Med.,' vol. 25, p. 488.



2



3







7



8



1





*Cellular Individuality in the Higher Animals, with Special Reference to the Individuality of the Red Blood Corpuscle. (II)*

By CHARLES TODD, F.R.S.

(Received August 18, 1930.)

(From the National Institute for Medical Research, Hampstead, London.)

In experiments described in an earlier communication (Todd, 1930), dealing with the examination of the red blood corpuscles of the domestic fowl by means of artificially prepared iso-agglutinating sera, it was found possible, by the use of such sera, to differentiate the corpuscles of any particular fowl from those of any other individual of the same species, provided that the individuals in question were not close blood relations.

In the case of closely related fowls, on the other hand, this could not always be done, at any rate by means of a single serum, as the red cells of certain members of a family were found to resemble one another so closely in their behaviour to the serum as to be indistinguishable from one another.

In order to investigate this point more fully the corpuscles of the chicks of three families of Plymouth Rock birds, specially bred for the purpose, were examined by means of polyvalent iso-agglutinating sera, exhausted with the corpuscles of the respective parents. In this way interesting relations were found to exist between the corpuscles of the parents and those of certain of their offspring but, as the chicks were then too small to yield an adequate amount of blood for exhaustion experiments, the action of sera exhausted for the corpuscles of the chicks could not be studied, and further work had to be postponed for a time. With the growth of the chicks it has now been possible to continue the investigation and, by means of suitably exhausted sera, to compare the characters of all the members of the three families; but the present communication is concerned mainly with the relations existing between the corpuscles of brothers and sisters. The methods employed were the same as those previously described (1930).

*Exhaustion of Serum with the Corpuscles of the various Members of a Family.*

It was necessary in the first place to prepare a specially exhausted serum for each member of the family under investigation. This was done by mixing a convenient volume (about 4 c.c.) of a highly polyvalent iso-agglutinating



serum with about one-fifth of its volume of the washed red cells of the fowl for whose corpuscles the serum was to be exhausted and allowing the mixture to stand for at least 15 minutes at room temperature, when it was centrifuged and the supernatant serum pipetted off. The procedure was repeated until the serum no longer showed any traces of agglutination when mixed with an equal volume of a 5 per cent. suspension of the corpuscles and rocked on a glass plate for 15 minutes. When this point was reached one further exhaustion was made, as a matter of precaution, and the serum stored at 0° C. until required for use.

This process was carried out for each member of the three families, so as to obtain a separate, specifically exhausted serum for each individual.

#### *Examination of Corpuscles of the Members of a Family.*

In order to study the relations existing between the corpuscles of the various members of a family, the agglutinating action of a serum exhausted for one of the members was tested separately on the corpuscles of each of the others, and the same process repeated for each of the exhausted sera. The agglutination tests were made, as previously described, by mixing equal volumes of the serum and of a 5 per cent. suspension of the red blood cells on a glass plate and rocking for 15 minutes; but in these large tests, in order to economise the exhausted sera, only one drop of each constituent was used—the areas of the squares on the glass plate being correspondingly diminished—and a simple mechanical arrangement was employed for rocking the plate.

The process of bleeding the fowls, exhausting the sera, and testing the corpuscles of so large a number of birds naturally took a considerable time, so that the tests were spread over several weeks. It was thus not practicable to make an exact titration of the agglutinating power of the sera, but the degree of agglutination was recorded, and in cases where complete agglutination took place the time after which this occurred was noted, and was found to give a sufficiently good indication of the strength of the exhausted sera.

The general tests of the corpuscles of the three families have therefore no pretensions to any high degree of accuracy. In cases where this was desirable quantitative tests were subsequently made, these being carried out in parallel, on the same plate, and under more carefully controlled conditions.

The results for the three families—each comprising the two parents with 17, 18 and 13 chicks respectively—are shown in Tables I, II and III. In order not to complicate the tables the times taken for complete agglutination have been omitted.

Table I.—Examination of the Corpuscles of Members of a Family of Plymouth Rock Fowls (Red Family) by means of specifically Exhausted Iso-agglutinating Sera.

Immune serum exhausted with corpuscles of	Tested on corpuscles of															
	F	M	2	3	4	5	28	30	31	32	33	48	49	50	51	62
Father	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mother	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Chick	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
No. 2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
31	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
32	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
48	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
49	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
51	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
62	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
63	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
64	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
65	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

C = complete agglutination.

a.c. = almost complete agglutination.

+ = very marked agglutination.

++ = marked agglutination.

+++ = trace of agglutination.

— = complete absence of agglutination





*Individual Differences in the Corpuscles of Members of the same Family.*

On examining these tables in detail, and comparing the behaviour of the corpuscles of the individual members in any one family, it will be seen that the cells of no two chicks appear to be exactly alike, but always to show some degree of difference, which may vary from a close resemblance to a very marked contrast.

In some cases, it is true, the corpuscles of several chicks may resemble one another so closely that the question at once arises as to whether the small differences are not to be attributed to experimental errors in determining the degree of agglutination, and, in view of the difficulty in exactly titrating an agglutinating serum, this question must receive consideration. Attempts to settle the point were made by repeating the tests—in the case of a number of the chicks which showed the greatest resemblances—making these on the same plate and as far as possible under the same conditions. This was done for chicks from each of the three families. In no case were the corpuscles of two chicks found to give exactly similar results when tested with a series of exhausted sera; but it must be frankly recognised that too great reliance cannot fairly be placed on agglutination tests, where certain factors, such as the exact strength of the corpuscular suspension, are not easily controlled, and the end-point of the reaction is not absolutely sharp.

In the majority of the chicks, however, the behaviour of their corpuscles to the exhausted sera shows unmistakable differences, and in many cases these are very striking.

*Immunisation of Chicks with the Corpuscles of their Brothers or Sisters.*

Considering the very marked individual differences shown by the red cells of the chicks in any one family, it appeared not unreasonable to attempt the immunisation of chicks with the corpuscles of their own brothers or sisters, and for this purpose four pairs of chicks were chosen from the same family (Table III, blue family), and one member of each pair was immunised with the corpuscles of the other.

In the first pair (No. 14 and No. 45) the corpuscles of the two birds were very definitely different in character, as shown by the fact that a serum exhausted for the cells of either bird still actively agglutinated the cells of the other, and by their different behaviour when tested with a series of exhausted sera.

In the second pair (No. 15 and No. 16) also, the corpuscles of neither of the members exhausted a serum for the corpuscles of the other, but the difference between the cells was not quite so pronounced as in the case of the first pair.

The third and fourth pairs (Nos. 13 and 17, and Nos. 43 and 319) were composed of chicks specially selected as having corpuscles which could only be distinguished with difficulty, and without any degree of certainty, by means of the exhausted sera.

The birds were immunised by doses of 15 c.c. of fresh citrated blood given intramuscularly at intervals of a week ; the serum of the injected birds being tested after an interval of 7 days after each injection of blood.

The results of the experiment are shown in the following table :—

Table IV.—Immunisation of Chicks of the same Family with the Blood of their Brothers or Sisters.

Chick immunised.	Blood used for immunisation.	Resemblance or otherwise between the corpuscles of the two chicks.	Result
No. 14	No. 45	Corpuscles very definitely different. Do not exhaust for one another.	Agglutinin formed after 2 injections.
„ 16	„ 15	Corpuscles definitely different. Do not exhaust for one another.	Agglutinin formed after 4 injections.
„ 17	„ 13	Corpuscles almost indistinguishable. Exhaust for one another.	Agglutinin formed after 8 injections.
„ 319	„ 43	Corpuscles almost indistinguishable. Exhaust for one another.	No agglutinin formed after 10 injections.

Close blood relationship between the injected fowl and the bird furnishing the corpuscles is therefore, in itself, no bar to the formation of iso-agglutinins, but it would appear that the ease with which these are formed is dependent upon the degree of difference in the characters of the cells of the two individuals.

In the first two pairs, whose corpuscles were very different in character, the formation of antibodies took place quite quickly ; but in the third pair, where the corpuscles of the two birds were almost indistinguishable, the appearance of agglutinins did not occur until after the eighth injection.

In the case of the fourth pair, even ten injections of blood were not sufficient to produce the necessary stimulus for antibody formation.

The agglutinating sera obtained in this way in closely related birds were highly specific, and only acted on corpuscles resembling those used for their production, but for these cells they appeared to be as active as the sera prepared in non-related fowls.

*Discussion.*

There can be little doubt that the red blood corpuscle must be regarded as a "multiple antigen," in the sense that it contains a large number of different antigenic units or "receptors," each of which when introduced parenterally into a suitable individual, is capable of giving rise to the production of a corresponding antibody, and all the experimental evidence appears to indicate that these antigenic units behave independently of one another.

It seems clear too, on comparing the characters of the cells of the offspring with those of their parents, that the antigenic units of the corpuscles maintain their independence during the process of hereditary transmission. This fact is of interest, as suggesting a possible method of attacking certain problems of genetics in the higher animals. Up to the present attention has here necessarily had to be directed in general to an investigation of the hereditary transmission of certain more or less easily recognisable characters, such as the size, form, and colouring of the animal. The number of individual differences which can be recorded in this way is naturally limited, and the characters studied are, moreover, comparatively gross and often complex in nature, involving as they do a number of cells of different structure and function, so that they can hardly be regarded in any real sense as unit characters.

By means of the immune iso-antibodies, on the other hand, it is possible—at any rate in the case of the red blood cell—to investigate isolated cells of a single type, without the complicating presence of other body-cells.

It is true that the red blood corpuscles have been utilised for the study of heredity by a number of observers, who have investigated the distribution of the four naturally occurring groups of human blood cells in a very large number of families, and it has been shown that these groups are racial, and that their inheritance follows the Mendelian laws. These group differences are, however, on an entirely different plane from the immeasurably finer differences which can be demonstrated by means of the artificially produced iso-antibodies.

*Conclusions.*

1. When a family of Plymouth Rock fowls is examined by means of exhausted immune iso-agglutinating sera, the corpuscles of no two chicks appear to be exactly alike; the cells of different individuals show degrees of difference in immunological behaviour, which may vary from a close resemblance to a very marked contrast.

2. Individual chicks may be immunised with the blood of their own brothers or sisters and yield active but highly specific agglutinating sera.

3. The ease with which such agglutinins are formed seems to vary with the degree of difference in character between the corpuscles of the injected fowl and those used for immunisation.

4. The red blood corpuscle must be regarded as a "multiple antigen" in the sense that it contains a large number of different antigenic units or "receptors," which apparently behave as independent units when hereditarily transmitted.

The writer wishes to record his indebtedness to the Medical Research Council, to Capt. S. R. Douglas, F.R.S., in whose department of the Institute the work was carried out, and to Major G. W. Dunkin, M.R.C.V.S., who very kindly supervised the breeding and care of the fowls.

#### REFERENCE.

Todd, C. (1930). 'Proc. Roy. Soc.,' B, vol. 106, p. 20.

543.849:547.828

### *A Method for the Estimation of Iron in Biological Material.\**

By ROBERT HILL, Beit Memorial Research Fellow.

(Communicated by Sir Frederick Hopkins, F.R.S.—Received August 5, 1930.)

(From the Biochemical Laboratory, Cambridge.)

#### I. *Introduction.*

The intense red colour and the stability of the product of reaction between ferrous salts and  $\alpha\alpha'$  dipyridyl has remained a striking instance of complex salt formation since the discovery of this dipyridyl by Blau (1888). Although for obvious reasons this compound would seem a good reagent for the colorimetric estimation of iron, it has never been used to any extent for the purpose, without doubt because there are other more sensitive and accessible reagents. It seems, however, to possess unique advantages for the study of iron in connection with living material, and on this account it was thought advisable to bring  $\alpha\alpha'$  dipyridyl, as a reagent for iron, to the notice of biologists.

\* Part of this work was carried out during the tenure of a senior studentship of the Exhibition of 1861.



$\alpha\alpha'$  dipyridyl was first prepared by Blau (1888) by distilling the copper salt of  $\alpha$  picolinic acid; the yield is small, but this method has the advantage of readily yielding a pure product. Recently Hein and Retter (1928) have found a relatively simple method of preparation by heating pyridine in a sealed tube with an oxidising agent such as ferric chloride, which removes hydrogen without the formation of water. This method though more economical needs greater manipulation to produce the substance in a state of purity. Smith (1926) isolated the same substance by the oxidation of sodium pyridines with dry air above  $100^{\circ}\text{C}$ . The ferrous complex salts were examined by Werner (1912) who showed that they could be resolved into two optical isomers. The ferrous salts of  $\alpha\alpha'$  dipyridyl have the formula  $[\text{Fe}(\text{C}_{10}\text{H}_8\text{N}_2)_3]\text{X}_2$  (Blau, 1898) where X is a monovalent acid radicle, and from the fact that the optical isomers do not racemize at once (Werner, 1912) dissociation into free ferrous salts must be relatively slight. They differ from the normal ferrous salts in not being easily oxidised, in fact only powerful agents such as permanganate or chlorine oxidise them to a blue compound. This latter compound, which should be the ferric complex salt, is not obtained directly from ferric salts and  $\alpha\alpha'$  dipyridyl and passes very easily into the red ferrous salt.

These properties at once suggest the use of dipyridyl not only as a reagent for detecting iron in the presence of reducing agents where other reagents are unsuitable but as a reagent for inhibiting the catalytic reactions of iron. It is from these considerations that the experiments described in this paper have been carried out.

## II. Preparation of $\alpha\alpha'$ Dipyridyl.

The method based on that of Blau (1888) was adopted. 45 g. of  $\alpha$ -picoline (boiling point  $126\text{--}132^{\circ}\text{C}$ .) were heated under a reflux condenser with 173 g. of potassium permanganate dissolved in 4.25 l. of water for 7 hours (Weidel, 1879). The colourless liquid was filtered from the hydrated oxides of manganese and the alkalinity reduced by passing in a current of  $\text{CO}_2$ . The bulk of the water was then distilled off together with the unchanged bases and the residual fluid evaporated to dryness over a water bath. The residue was dissolved in 150 c.c. of water and filtered. Glacial acetic acid was then added until the liquid was acid ( $p_{\text{H}}$  5-6) and then about 180 c.c. of 1 per cent. copper sulphate solution was added slowly until there was no further precipitation of the blue-violet crystals of copper picolinate. The copper salt was filtered off after 15 minutes, washed with cold water in which it is sparingly soluble and dried, first at room temperature, then at  $105^{\circ}\text{C}$ .; 40 g. were obtained.

The dry copper picolinate was destructively distilled in a slow current of dry  $\text{CO}_2$  from a retort, the receiver being cooled in ice, until a dry blackish residue was left. The liquid distillate, about 15 c.c., containing  $\alpha\alpha'$  dipyridyl and pyridine was heated to  $50^\circ \text{C}$ . on a water bath and a current of air blown through until the pyridine had been largely removed. The residue which solidified on cooling was then distilled with steam after the addition of a few drops of 40 per cent. soda. 1.4 g. of  $\alpha\alpha'$  dipyridyl were obtained on cooling the distillate. The distillation was then continued until the fluid gave only a faint colour with ferrous sulphate. This distillate added to the previous mother liquor was evaporated with an excess of hydrochloric acid to dryness on a water bath. The residue was dissolved in a little water, made alkaline with soda and distilled with steam. 1.2 g. were obtained. The  $\alpha\alpha'$  dipyridyl was obtained as colourless shining plates, melting point  $71^\circ \text{C}$ . (when dried over  $\text{KOH}$ ). It is sparingly soluble in water; the saturated solution at  $20^\circ \text{C}$ . is 0.025 molar and has a  $p_{\text{H}}$  of 7.2. It is appreciably volatile at ordinary temperature and the vapour has an aromatic odour recalling that of vanillin. It is very stable to acids and alkalies and is unaffected by mild oxidising and reducing agents.

### III. *Method of Estimation of Iron.*

On adding  $\alpha\alpha'$  dipyridyl to a solution of a ferrous salt between  $p_{\text{H}}$  3.5 and 8.5 the intense red complex ferrous ion is produced. The presence of other metals unless in great excess over the iron does not influence the colour. For instance, a copper salt gives a blue colour which is nearly invisible at a concentration of copper 1000 times that of the concentration of iron needed to render the pink colour just perceptible. Zinc on the other hand gives a colourless compound sparingly soluble in water (Blau, 1889). If other heavy metals are present in appreciable quantities it is necessary to use an excess of the reagent, when testing for iron, so that all the metals may be combined with the dipyridyl.

Ferric iron unless present in high concentration gives no colour and does not interfere with the reaction with ferrous iron. Thus it is a simple matter by adding dipyridyl and measuring the intensity of the pink colour before and after reducing the iron to estimate ferrous and ferric iron in a mixture.

The ferrous dipyridyl salts are soluble in the presence of most anions. They are less soluble in the presence of an excess of iodide and are precipitated by tungstates and the usual alkaloidal reagents. If pyrophosphates are present, owing to the stability of ferric pyrophosphate, it is necessary to use a reducing agent and as long as the iron is kept in the ferrous state pyrophosphate does

not influence the colour. Thiocyanates, while giving the intense red colour with ferric iron, give no colour with ferrous iron and do not affect the ferrous dipyridyl salts.

$\alpha\alpha'$  dipyridyl combines, however, with ferric thiocyanate in presence of HCl giving an intense bluish-red substance insoluble in water and only sparingly soluble in ether and amyl alcohol. With a copper salt and thiocyanate in the presence of acid  $\alpha\alpha'$  dipyridyl similarly gives a green-yellow substance insoluble in both aqueous and organic solvents.

If  $\alpha\alpha'$  dipyridyl is added to reduced hæmatin there is no change in colour or absorption spectrum; the same is true if it is added to pyridine hæmochromogen. If hæmatin is precipitated with acetate buffer in the presence of dipyridyl no colour can be obtained in the supernatant fluid on adding reducer. This shows that the iron of hæmatin is not removed by dipyridyl.

It therefore follows that to estimate the inorganic iron in a fluid between  $p_H$  3.5 and 8.5 all that is required is to add dipyridyl and a reducing agent, and determine the intensity of the pink colour.

#### IV. *Purification of Sodium Hydrosulphite.*

For a reducing agent on the acid side of neutrality sodium hydrosulphite seems most suitable. As this substance always contains iron it must be purified before use. This is accomplished most easily by the following method. The sodium hydrosulphite is dissolved in warm water (40° C.) to give a nearly saturated solution and a small quantity of  $\alpha\alpha'$  dipyridyl added so that the maximum red colour is obtained. The dark red solution is then rapidly filtered on a Buchner funnel and the salt precipitated from the filtrate by alcohol. After standing 20 minutes the salt is filtered off and washed with 70 per cent. alcohol till colourless, then with 97 per cent. alcohol. The solid is then boiled with 97 per cent. alcohol for 10 minutes, filtered off at once while hot and immediately transferred to a vacuum desiccator containing sulphuric acid. This preparation was found to keep well and to give no red colour either with iron or  $\alpha\alpha'$  dipyridyl, and if the manipulations are conducted with reasonable speed there is no serious oxidation of the hydrosulphite during its purification.

#### V. *Preparation of Standard Iron Dipyridyl.*

For the estimation of the pink colour it is most convenient to use a series of standard tubes similar to those used for the determination of  $p_H$  by means of indicators. By this method it is possible to determine the iron with an

accuracy of 10 per cent. down to a concentration of 0.0003 mg. Fe per cubic centimetre. The standard tubes are most conveniently made up as follows. Stock solutions of M/100 ferrous iron (0.392 g. ferrous ammonium sulphate in 100 c.c.) and 3M/100  $\alpha\alpha'$  dipyridyl hydrochloride (0.468 g. of  $\alpha\alpha'$  dipyridyl and 6 c.c. of N. HCl in 100 c.c.) were prepared. These solutions were then equivalent from the point of view of the coloured complex ion. A solution of iron dipyridyl M/10,000 in acetate buffer was prepared containing twice the theoretical amount of  $\alpha\alpha'$  dipyridyl and a trace of sodium hydrosulphite. 5 c.c. of this solution were taken as the first of the series of standards, and the others made up as follows. For preparing 5 c.c. of a standard the formula  $x = 5f^{(n-1)}$  was used, where  $x$  is the number of cubic centimetres of the M/10,000 solution of iron dipyridyl,  $f$  is a factor for the dilution, and  $n$  is the number of the standard, the fluid being made up to 5 c.c. with acetate buffer in each case. The factor was 19/21; this gives a difference equal to 10 per cent. of the mean iron concentration of two consecutive standards. For example, in the case of standard No. 2,  $x = 5 \times \frac{19}{21}^{(2-1)} = 4.52$ ; buffer to be added = 0.48 c.c. Standard No. 1 contains 0.0056 mg. Fe per cubic centimetre and standard No. 2 0.0051 mg. Fe per cubic centimetre. By this method a series of 24 standards was prepared giving a range of concentration of iron from 0.0056 mg. per cubic centimetre to 0.00056 mg. Fe per cubic centimetre, the limits found most suitable for general use. These were sealed off in tubes 1.3 cm. in diameter and 8 cm. long containing 5 c.c. of the fluid. To ensure the greatest permanence some SO<sub>2</sub> should be passed through the liquid to convert a little of the acetate into sulphite. Prepared in this way a standard of M/10,000 Fe made 3 years ago was found to be unaltered on comparing it with a fresh standard.

#### *VI. Effect of $p_H$ on the Colour given by Ferrous Salts and Dipyridyl.*

On adding a dilute mineral acid to a dilute solution of the iron dipyridyl salt the colour slowly fades, showing that acids dissociate the complex. The same effect is produced by adding alkali. In order to define the limits of  $p_H$  suitable for the estimation of iron some experiments were carried out in buffer mixtures, the concentrations of dipyridyl iron being measured by the standard tubes. On the acid side of neutrality glycine HCl and phthalate buffers were used.  $10^{-4}$  M. iron was present and the theoretical amount of dipyridyl (3 times  $10^{-4}$  M.). The experiments on the association and dissociation of the complex were in agreement, equilibrium being established in 10 to 15 hours. The dissociation followed a rather steep curve extending from  $p_H$

1.7 to  $p_H$  3.7 where there was nearly complete association. The presence of excess of  $\alpha\alpha'$  dipyridyl shifted the curve along the scale to a more acid range. As  $\alpha\alpha'$  dipyridyl is appreciably soluble in water an excess can always be present when using it as a reagent. Hence on the acid side of neutrality it is safe to reach  $p_H$  3.5. On the alkaline side owing to the complication of the oxidation and precipitation of the iron it was not possible to obtain precise results. Some experiments were made in glycine NaOH buffers and cysteine used as a reducing agent. At a concentration of  $10^{-4}$  M. Fe the dissociation extended from  $p_H$  8.5 to  $p_H$  10. Hence on the alkaline side of neutrality it is safe to reach  $p_H$  8.5.

The range of  $p_H$  is thus from 3.5 to 8.5, when an excess of dipyridyl is used. For controlling the  $p_H$  it is usually sufficient to add to the fluid to be tested a solution of sodium acetate (free from iron) containing a little acetic acid, as the iron compound shows its maximum stability throughout the range of the acetate buffer mixtures. The iron compound can be detected up to  $p_H$  8.5 if a slight excess of dipyridyl is added and hydrazine hydrate used as a reducer at  $40^\circ$  C.

#### *XII. Adsorption of Dipyridyl Iron by Insoluble Proteins.*

Werner (1912) pointed out that iron dipyridyl will dye animal fibres red. It will not dye vegetable fibres and is not adsorbed by filter paper to an appreciable extent. It is, however, adsorbed by proteins hence when estimating iron in the presence of insoluble proteins care must be taken that the iron is not lost through adsorption. In the case of fresh yeast the adsorption of added dipyridyl iron was found to be negligible. In the case of boiled yeast, there was about 50 per cent. adsorption of the iron dipyridyl at  $p_H$  6, but by passing  $SO_2$  in the presence of acetate until the  $p_H$  was 4 the adsorption was very slight and the colour could easily be removed by washing. The same was found to hold in the case of the proteins of boiled egg yolk. In 30 per cent. alcohol the iron compound is readily soluble and shows no adsorption on either animal fibres or insoluble proteins if the fluid is acid; so that if the method of extraction of the iron dipyridyl with acetate and sulphite fails alcohol can be used. In neutral or slightly alkaline solution the adsorption on the proteins is very marked and the iron compound is not always extracted even by alcohol.

#### *XIII. Estimation of the Inorganic Iron in Bakers' Yeast.*

20 g. of bakers' yeast were washed with glass distilled water until the washings gave no colour with dipyridyl and sodium hydrosulphite. The yeast was

then suspended in dilute acetate buffer and dipyrldyl added. No colour was produced on standing 15 minutes. A little iron-free sodium hydrosulphite was then added and the yeast immediately became pink. Hence in the washed yeast the iron is practically all in the ferric state. This is also true of unwashed yeast. The yeast was then washed until the liquid was colourless. The remaining residue of pink yeast cells was suspended in dilute acetate buffer and after the addition of a little more  $\alpha\alpha'$  dipyrldyl and hydrosulphite  $\text{SO}_2$  was passed through the suspension. This caused apparent damage to the cell membranes, because the colour diffused out into the liquid leaving the yeast practically colourless. The yeast was then washed. The iron dipyrldyl extracted in these three stages was made up to 100 c.c. in each case and the solutions reduced by adding hydrosulphite (to bleach certain yellow substances of the yeast cells). The iron was estimated colorimetrically with the standard tubes, and the results were as follows, the iron being expressed as a percentage on the original wet weight of the yeast. Washings, 0.00012 per cent. ; removed from the yeast by dipyrldyl, 0.00015 per cent. ; extracted after damaging the cell membranes, 0.0013 per cent. ; total 0.0016 per cent. It appears therefore that about 80 per cent. of the inorganic iron in this sample of yeast was actually inside the cells in the ferric form. The remaining 20 per cent. being due to some dead cells and chance contaminations.  $\alpha\alpha'$  dipyrldyl in sodium hydrosulphite at  $p_{\text{H}}$  4 does not easily liberate the iron from hæmatin compounds, and as the intracellular hæmatin compounds (Keilin, 1925) remain in the yeast during the manipulations, the iron found by  $\alpha\alpha'$  dipyrldyl should represent the non-hæmatin iron of the yeast.

#### IX. *Estimation of Iron in Egg Yolk.*

When egg yolk boiled or unboiled is suspended in acetate buffer and  $\alpha\alpha'$  dipyrldyl added, no red colour is produced, but on the addition of sodium hydrosulphite a deep red colour is immediately developed. This would indicate that the iron is present as free ferric iron rather than in any stable organic combination in the yolk. Bunge (1885) drew attention to the fact that the iron of egg yolk is not shown easily by the usual tests, but as the iron is so easily "unmasked" by a reducing agent, it would suggest that in the egg yolk we have colloidal ferric hydroxide. The iron was therefore estimated in the yolk of an egg (hen) by means of dipyrldyl with and without incineration. For extracting the iron from yolk of egg without ashing the following method was used. An egg was boiled 15 minutes and cooled in water. The yolk was broken up into a homogeneous crumbly mass and 1 g. samples immediately

weighed out. One sample was suspended in acetate buffer (5 c.c.) and treated with  $\alpha\alpha'$  dipyridyl and sodium hydrosulphite. After passing  $\text{SO}_2$  until the proteins and fat were aggregated the mixture was filtered and the filtrate and washings made up to 50 c.c. Another sample was treated in a similar way, alcohol being used (30 per cent.) instead of  $\text{SO}_2$  to prevent adsorption of the iron compound on the protein. Two further samples were incinerated with 5 c.c. concentrated sulphuric acid and 1 g. of potassium perchlorate (the iron content of which was determined) added with due precautions. After incineration water was added and the solution was neutralised with sodium acetate and made up to 100 c.c. with the addition of sodium hydrosulphite and dipyridyl. The iron was determined in solution by the standard tubes. The results were as follows: by incineration the iron content was found to be 0.0086 and 0.0083 per cent. of wet weight; without incineration, using  $\text{SO}_2$ , 0.0080 per cent.; and using 30 per cent. alcohol, 0.0085 per cent. In order to see if the iron was liberated from some organic combination by acid and a reducing agent a small quantity of the egg yolk was suspended in 10 per cent. ammonia solution and warmed with a little dipyridyl and hydrazine hydrate. The mixture became red showing that the iron is "unmasked" even by a reducing agent in alkaline solution.

*X. The Effect of  $\alpha\alpha'$  Dipyridyl on the Iron and Copper Catalysis of the Oxidation of Cysteine at  $p_{\text{H}}$  7.3.*

In each experiment a series of four separate Barcroft differential manometers was used, each containing in the right-hand side 5 mg. cysteine hydrochloride (neutralised), 1 c.c. of phosphate buffer, the total volume of fluid being 3 c.c. The left-hand side contained 3 c.c. of buffer. The control contained the maximum quantity of  $\alpha\alpha'$  dipyridyl used in an experiment and no metals. The temperature was 20° C. In the following table are shown the results of two experiments. The cysteine without metal absorbed about 4 mm.<sup>3</sup> of oxygen per hour.

Iron added = 0.0033 mg.

Molecular ratio $\frac{\text{dipyridyl}}{\text{metal}}$	oxygen uptake mm. <sup>3</sup> per hour.
Zero	119
3	52
6	Zero

Copper added = 0.0013 mg.

Zero	263
3	278
6	238

It is seen, as might have been expected, that  $\alpha\alpha'$  dipyridyl in low concentration inhibits the catalysis by iron. It is, however, curious that while the colormetric measurements indicate complete association of iron and dipyridyl at  $p_H$  7.3 (cysteine not influencing the colour) only about 50 per cent. inhibition is observed in the presence of the theoretical amount of dipyridyl. There is no appreciable effect on the copper catalysis at this range of dipyridyl concentration.  $\alpha\alpha'$  dipyridyl thus behaves in a corresponding way to pyrophosphate, only it shows its effect at a lower concentration comparable with that required in the case of cyanide inhibitions.

#### XI. *Summary.*

1.  $\alpha\alpha'$  dipyridyl is proposed for a reagent for estimating inorganic iron in biological material for the following reasons :—

- (a) The colour is almost specific for ferrous iron, other metals such as copper giving but faint colours.
- (b) There is no necessity for ashing, while other methods require it.
- (c) The standards can be kept indefinitely and the fluid after adding dipyridyl for estimation can be kept without fear of the colour fading.
- (d) The colour is given quantitatively over a region of  $p_H$  which covers the physiological range.
- (e) It is possible to determine the ferrous and ferric iron in a mixture.
- (f) The colour does not become yellowish in great dilution.
- (g) The colour is stable to reducing agents such as sodium hydrosulphite.
- (h) The sensitivity approaches that of the thiocyanate method, it being possible to detect iron in a concentration of 0.0002 mg. per cubic centimetre in a layer of fluid 1.3 cm. thick.
- (i) Hæmatins and their various compounds do not give a reaction for iron with  $\alpha\alpha'$  dipyridyl.

2. The inorganic iron in yeast and egg yolk appears to be in the ferric form, and that in egg yolk resembles colloidal ferric hydroxide in properties, there being no detectable organic iron analogous to a hæmatin compound.



3.  $\alpha\alpha'$  dipyridyl resembles pyrophosphate in its inhibition of iron and not of copper in the oxidation catalysis of cysteine by these metals.

## REFERENCES.

- Blau (1888). 'Ber. D. Chem. Ges.,' vol. 21, p. 1077.  
Blau (1889). 'Mösch. Chem.,' vol. 10, p. 375 (from Werner).  
• Blau (1898). 'Mösch. Chem.,' vol. 19, p. 647 (from Werner).  
Bunge (1885). 'Z. physiol. Chem.,' vol. 9, p. 49.  
Hein and Retter (1928). 'Ber. D. Chem. Ges.,' vol. 61, p. 1790.  
Keilin (1925). 'Proc. Roy. Soc.,' B, vol. 98, p. 312.  
Smith (1926). 'J. Amer. Chem. Soc.,' vol. 48, p. 416.  
Wiedel (1879). 'Ber. D. Chem. Ges.,' vol. 12, p. 1992.  
Werner (1912). 'Ber. D. Chem. Ges.,' vol. 45, p. 433.

612.742

*The Heat of Rigor of Mammalian Muscle.*

By E. C. SMITH, Low Temperature Research Station, Cambridge.

(Communicated by Sir William Hardy, F.R.S.—Received August 6, 1930.)

The stiffening of muscle in *rigor mortis* is closely related to gelation of the muscle plasma (Smith, 1930). Neither the stiffening of the muscle (Hoet and Marks, 1926) nor the gelation of the plasma is immediately due to an increase in the hydrogen-ion concentration of the muscle, but, apart from the formation of lactic acid, no reaction is known to occur *post-mortem* which might be held responsible for the coagulation of the plasma. It was with a view to the detection of any such reaction that the following measurements of the heat production accompanying *rigor mortis* were made.

The heat of *rigor mortis* has not previously been measured, although A. V. Hill (1912) measured the heat produced by frog's muscles undergoing heat and chloroform rigor. The result suggested that the conversion of glycogen into lactic acid accounted for almost the whole of the heat produced. This has been found to be the case in the muscle of a normal well-fed rabbit when passing into *rigor mortis*, and also in the case of fatigued or exhausted muscle, but starved animals produce a larger quantity of heat than can be accounted for by the lactic acid produced.

Evidence of chemical changes other than the breakdown of glycogen has recently been obtained by McCullagh, Meyerhof and Schulz (1930). These changes are most marked in the early stages of anaerobiosis in amphibian muscle, and it is possible that the unknown source of heat in the following experiments is identical with that described by these authors.

*Methods.*—The heat production was measured by the rise in temperature of the muscle kept in a Dewar flask immersed in a water bath at constant temperature ( $21^{\circ}\text{C}.$ ). The flask was closed with a rubber bung bearing delivery tubes for replacing the air of the flask with hydrogen. Measurements of temperature were made by means of a Beckmann thermometer.

The experiments were required to afford a comparison of the rate of heat production with the rate of stiffening of the muscle and the rate of gelation of the plasma, consequently a large mass of muscle had to be used. The whole of the thigh and calf muscles of the limb of a rabbit were used for each experiment.

The animals were killed by a blow on the head, and the muscles of the lower half of the body were brought rapidly to the temperature of the bath by perfusion through the abdominal aorta with ice-cold Ringer solution. A needle thermocouple was used to register the temperature at various points in the interior of the muscle during this operation. The limb was severed at the hip joint by a clean cut with a sharp knife, and the foot removed above the ankle. The thermometer was inserted in the space between the adductor and vastus muscles, the leg doubled, and thrust into the bottom of the flask. The thermometer was then imbedded in the centre of the mass of muscle and served to hold the muscle firmly whilst the flask was evacuated. The evacuation was effected as rapidly as possible so that loss of heat by evaporation was minimal, and the flask was then filled with hydrogen, which had been purified from oxygen by passing over platinised copper turnings at  $400^{\circ}\text{C}.$

After the flask had been placed in the bath, 1 hour was allowed for the temperature to become even throughout the muscle. It was desirable that the initial temperature of the muscle should be about half a degree below the bath temperature, and with the method of preparation adopted this was usually the case. Since the rise in temperature of the muscle was of the order of  $1^{\circ}\text{C}.$  the correction for loss or gain of heat from the surroundings was then minimal.

The measurement of temperature continued from 8 to 12 hours, at the end of which time heat production practically ceased.

*Determination of the Cooling Coefficient of the Calorimeter.*

The cooling coefficient of the calorimeter was determined by observing the rate of rise or fall of temperature of a similar quantity of muscle which had been autoclaved at 120° C. Observations were made on muscle of an initial temperature both higher and lower than the bath temperature, and a mean of the results taken for calculating the cooling coefficient of the calorimeter.

*Water Equivalent of the Calorimeter.*

This was determined by the method of mixing. The total weight of water was of the same order as the weight of the muscle used in the experiments.

*Specific Heat of Muscle and Bone.*

Determinations were made by the method of mixing of the specific heat of the muscle and bone as used in the experiments. For perfused muscle alone, values of 0.875, 0.890, 0.855, 0.900, 0.892, 0.871; mean 0.880 were found. (For unwashed muscle, a mean of 0.870.) The specific heat of the bone was 0.48, 0.50; mean 0.49. For the whole limb the specific heat was 0.825.

*Calculation.*—The heat production per gram of muscle  $H$  is related to the observed rise in temperature  $\Delta t$ , by the formula

$$H = \frac{\Delta t [(M + m) 0.825 + W]}{M}$$

$M$  being the weight of the muscle,  $m$  of the bone, and  $W$  the water equivalent of the calorimeter.

*Lactic Acid.*

An estimate of the increase in concentration of lactic acid in the muscle during the period of heat production was made on the assumption that, 1 hour *post-mortem*, the lactic acid content of the muscle of the right hind limb was identical with that of the left, the two limbs being maintained at the same temperature after removal from the body. Within reasonable limits of error this is true (Table I). Since the final concentration reached was usually greater than 0.5 per cent. the error involved in this assumption was about 6 per cent. For these determinations the whole limb was used. The muscle was separated from the bone and rapidly cut into pieces of 1 to 2 grams. The proteins were precipitated with ice-cold trichloroacetic acid, and lactic acid determined in the filtrate in the manner described in a previous paper (Smith, 1929).

It was hoped, in the same way, to be able to correlate heat production with

diminution in glycogen content, but in two animals the initial concentrations of glycogen in the muscle of the two limbs were so widely different (see Table I) that it would have been impossible to draw any conclusions from such experiments.

Table I.—Lactic Acid and Glycogen Percentage after 1 hour at 21° C.

Experiment.	Concentration in muscle.	Right leg.	Left leg.
		Per cent.	Per cent.
1	Lactic acid	0.18	0.16
2	"	0.19	0.19
3	Glycogen	0.52	0.78
4	"	0.20	0.31

In Table II are given the results of four experiments in which the heat production was compared with the lactic acid formed during the period of 1 to 10 hours *post-mortem*. The animals were all full-grown rabbits, rested and well-fed.

In carrying out the actual experiment, the right leg was placed in an incubator at 21° C. at the same time that the left leg was placed in the calorimeter. After 1 hour the right leg was used for the determination of lactic acid and the reading of temperature commenced on the left. Later, the left leg was removed from the calorimeter and its lactic acid content determined.

Table II.—Caloric Quotient of Lactic Acid in *Rigor Mortis*.

Experiment.	Heat production cals. per g. of muscle.	Lactic acid, per cent.			Caloric quotient.
		Initial.	Final.	Difference.	
5	1.175	0.18	0.59	0.41	285
6	1.305	0.17	0.65	0.48	270
7	1.010	0.32	0.67	0.35	290
8	0.885	0.20	0.51	0.31	284

The figures in the last column represent the heat evolved per gram of lactic acid appearing during the experimental period, *i.e.*, the "caloric quotient" of lactic acid. The mean value, 282 cals., coincides with the value obtained by Meyerhof for the caloric quotient in the caffeine rigor of frog's muscle, and may be attributed in its entirety to the heat produced by the breakdown

of glycogen to lactic acid (180 cal.) and the neutralisation of the latter with base (105 cal. approximately).

Further experiments confirm the view that the heat produced in rigor is normally derived from this reaction alone. Exercise or fatigue reduces the glycogen content of muscle, and may ultimately bring about its complete disappearance. This result has been obtained by McKay by electrical stimulation of the muscles of decapitate animals. An attempt was made to accomplish this in the whole of the musculature of the hind limbs of decapitate cats, but without success. The glycogen was considerably reduced, but the response to stimulation ceased before complete exhaustion of glycogen was attained. This was thought to be due to the accumulation of lactic acid in the preparation. An experiment was, therefore, carried out on the perfused hind limbs, in which a copious flow of oxygenated blood was supplied to the muscles. The glycogen content, judging by the eventual lactic acid concentration reached, was reduced by stimulation to a lower level than in any of the decapitate animals, but it was not completely removed. The muscles of a cat were employed, and a litre of defibrinated sheep's blood used for the perfusion. The muscles were stimulated by means of a ring of silver wire surrounding the body in the lumbar region, and through a pad of cotton wool moistened with saline solution in contact with the under surface of the feet. Single induction shocks of 40 a minute were administered. After 2 hours the response had almost completely disappeared. The heat production in this exhausted muscle was 0.33 cal. per gram, the corresponding lactic acid formation 0.11 per cent., representing a caloric quotient of 300. Thus the exhaustion of the muscle entails a diminution in heat production commensurate with the diminished breakdown of glycogen.

A fatigued muscle passes more rapidly into rigor than a normal one. The curve of rigor could not be followed with the sclerometer in the same experiments as those correlating heat production with the breakdown of glycogen, but it was evident without its use that rigor had not set in to any marked extent 1 hour after death. Part at least of the stiffening process took place in the period during which the heat measurement was made, and it follows that this stage of the stiffening was not associated with any appreciable heat evolution other than that due to the breakdown of glycogen.

Any method of restraining the breakdown of glycogen in a muscle has the disadvantage that *rigor mortis* sets in immediately the circulation stops. This is true of the effect of insulin, strychnine, starvation or the more recently discovered effect of iodacetic acid (Lundsgaard, 1930). None of these methods

can be employed to investigate the production of heat during the stiffening process but in the absence of lactic acid formation. It was thought of interest, however, to determine whether the effects of chemical change could be observed in the muscles of animals treated with iodicetic acid. This substance has the effect of completely inhibiting the breakdown of glycogen in muscle.

A rabbit was injected with 0.15 g. per kilo of iodicetic acid, as the sodium salt, hypodermically in the lumbar region. The muscles became rigid within 20 minutes of death. From the end of the first hour *post-mortem* to the ninth hour there was no production of heat, and no formation of lactic acid. If, therefore, any reaction associated with a considerable output of energy, other than the breakdown of glycogen to lactic acid takes place *post-mortem*, it also is inhibited by iodicetic acid.

In all the experiments so far described there is no evidence of such a reaction. It was observed, however, on two occasions when animals were used which had been brought immediately from the dealer, and which, according to him, had not been fed for 24 hours, that the heat production was abnormally large in comparison with the increase in lactic acid. In one of these, experiment 12, the caloric quotient appeared to be greater than 1000. The reality of this effect is proved by the additional experiments 13 and 14, which were carried out on two rabbits starved for 48 hours.

Table III.—Experiments on Animals subject to Experimental Procedure.

Experiment.	Treatment of animal.	Heat production cals. per gm.	Lactic acid.			Caloric quotient.
			Initial.	Final.	Difference.	
9	Muscle stimulated	0.33	0.27	0.38	0.11	300
10	Injec. iodicetic acid	Nil	0.16	0.17	0.01	—
11	24 hours' starvation	0.99	0.16	0.40	0.24	420
12	" "	0.98	0.20	0.28	0.08	—
13	48 hours' starvation	1.02	0.22	0.44	0.22	463
14	" "	0.73	0.26	0.44	0.18	405

The excess heat production (assuming a true caloric quotient for lactic acid of 282) in the four experiments was 0.31, 0.75, 0.39, 0.22 cals. per gram of muscle.

One can associate these observations with recent determinations by McCullagh, Meyerhof and Schulz (1930) of the caloric quotient of lactic acid in the anaerobic survival of frog's muscle. The excess heat which they find in the early stages

of anaerobiosis they are at present unable to correlate with any known reaction in muscle. It is too great to be accounted for by the breakdown of phosphagen, since this substance is not present in sufficient quantity in muscle for the purpose. These authors suggest that there are reactions occurring in muscle, accompanied by a considerable liberation of energy, the nature of which have yet to be discovered. It is of interest to find that one such reaction is associated with a condition of glycogen poverty in resting muscle.

*Comparison of the Rate of Stiffening with the Rate of Evolution of Heat.*

In general, both the process of stiffening and the evolution of heat in normal rabbit's muscle come to a standstill after about 8 hours *post-mortem* at 21° C. In any individual animal the course of heat production and stiffening can be followed simultaneously, using the sclerometer for the determination of the latter (*cf.* Smith, 1930).

Fig. 1 shows an actual result obtained in an experiment of this type. The

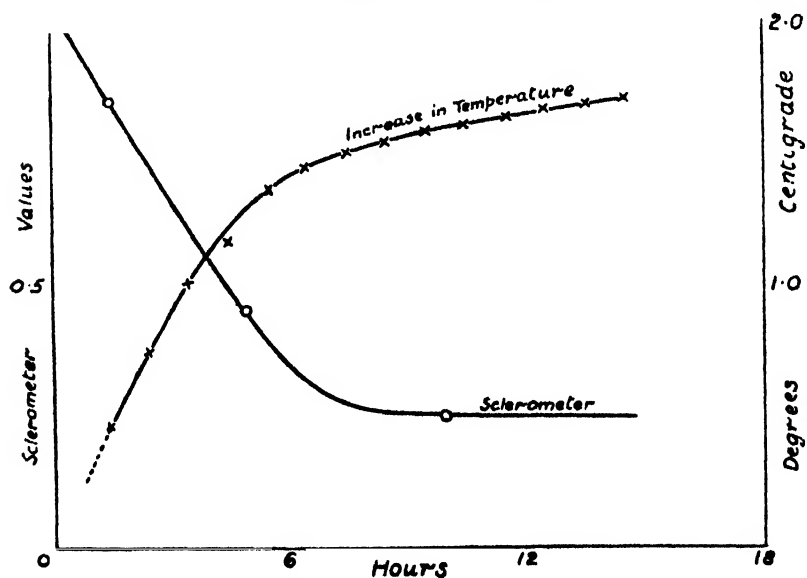


FIG. 1.—The coincidence between heat production and hardening of the muscle in *rigor mortis*.

course of stiffening is represented by the diminishing depression of the sclerometer as rigor proceeds. In general, there is a preliminary period of 1 to 2 hours during which no stiffening takes place, but occasionally, as in the instance represented, this period is omitted. From the commencement of stiffening

the process is continuous ; and after a variable time, usually between 7 and 10 hours, a degree of hardness is reached which is maintained until putrefaction sets in.

The course of heat production is continuous from 1 hour *post-mortem* until the seventh to the eighth hour. There is no reason to suppose that during the first hour the heat production is not equal to that in the succeeding hours, but accurate measurements could not be made in this period. From the end of the first hour, therefore, until rigor is completely established, stiffening and heat production follow exactly parallel courses.

The heat produced in the muscles of normal animals has been shown to be due entirely to the formation of lactic acid. It follows, therefore, that the stiffening in rigor is very closely associated with the accumulation of lactic acid in the muscle.

This conclusion appears to conflict with the evidence of Hoet and Marks on the onset of rigor in animals treated with insulin, with that of Lundsgaard on the effect of the injection of iodoacetic acid, and with the present author's view that changes in hydrogen-ion-concentration are irrelevant to the process of gelation in muscle plasma. In the complete absence of glycogen, stiffening occurs without appreciable formation of lactic acid, and is as severe as in the muscles of normal animals. In the muscles of animals treated with iodoacetic acid, the breakdown of glycogen is completely inhibited, yet the muscles are capable of stiffening. There is no reason to suppose that there is any fundamental difference between the stiffening in these and in normal animals.

The reasonable conclusion to be drawn is that the association which is here shown to exist between stiffening and lactic acid formation is an indirect one. It has, for instance, been suggested (Hoet and Marks, 1926) that the stiffening is associated with the disappearance of glycogen from the muscle, and, since lactic acid formation must vary directly with the breakdown of glycogen, a correlation between stiffening and removal of glycogen would satisfy the necessary condition that stiffening is directly proportional to the rate of formation of lactic acid. This explanation cannot be accepted, however, for rigor may be fully established in normal well-fed animals whilst a considerable residue of glycogen remains in the muscle.

It remains, then, to discover a third change to which both stiffening and lactic acid production are intimately related. It may be possible to identify this change with certain reactions which are already known, such as the hydrolysis of creatine phosphoric acid, but the recent work of Meyerhof and Lundsgaard has shown that still further possibilities of chemical



change exist, and it is as well to restrain speculation until more is known of these reactions.

## REFERENCES.

- Smith (1930). 'Proc. Roy. Soc.,' B, vol. 105, p. 579.  
 Hoet and Marks (1926). 'Proc. Roy. Soc.,' B, vol. 100, p. 72.  
 Hill (1912). 'J. Physiol.,' vol. 44, p. 466.  
 McCullagh, Meyerhof and Schulz (1930). 'Pflüger's Arch.,' vol. 224, p. 230.  
 Smith (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 198.  
 Lundsgaard (1930). 'Biochem. Z.,' vol. 217, p. 162.

612 . 17 : 599 . 32 Marmota.

*Observations on Certain Physiological Processes of the Marmot.*  
 I.—*The Heart.*

By G. ENDRES, B. H. C. MATTHEWS (Beit Fellow), H. TAYLOR and ALISON DALE (Michael Foster Student), Physiological Laboratory, Cambridge.

(Communicated by J. Barcroft, F.R.S.—Received July 3, 1930.)

[PLATES 17, 18.]

In the autumn of 1929 thirteen marmots were brought to Cambridge by one of us (Endres) after the hibernation period had commenced. Of these, we owe four to the kindness of Prof. Adolph Löwy of the Schweizer Forschungsinstitut at Davos. The remainder came from Roffna near the Julier Pass. During the first few days of their captivity in Cambridge they were kept at about 5° C. in the Low Temperature Station, and for this hospitality we would like to thank Sir William Hardy. This temperature proved too low. One of the most remarkable facts about marmots, which has been described by Mangili\* is that they can be awakened, not only by heat but by cold; at temperatures slightly above 0° C. several of the marmots woke. We shall have to refer to this phenomenon later. The marmots were then placed in an unheated wooden "Army hut," in a wooden box which was contained in a much larger box. Between the two boxes was sawdust. Also sawdust and hay were put in the small box. The smaller box was covered with a lid, and there was a hole in the side,

\* Cited by Dubois. "Physiologie comparée de la marmotte." Paris, 1896.

through which the marmots could leave the inner box for purposes of defæcation or micturition. It is stated in the literature that a marmot wakes up about once in three weeks for this purpose. Our experience was that, when the animals first came, they performed the functions of micturition and defæcation more frequently than at a later stage in the winter. They were losing water all the time, which was not replenished.

The temperature of the box containing the marmots was always above that of the outside air. During the winter of 1929-30 there was very little frost, and the lowest temperature noted on the "minimum" registering thermometer in the outer box was 2° C., and that only once.

### *The Temperature of the Marmots.*

The temperature of the marmots varies with that of the external air. It is known that during the act of waking the rectal temperature is not a reliable index of the body temperature. We were not prepared, however, for so great a difference as we observed on some occasions. The maximum disparity noted by us between the temperatures of the rectum and the heart was 17° C.; on one occasion a thermocouple placed well in the rectum registered 11° C., whilst another inserted into the heart registered 28° C. The thermocouple used for the heart consisted of a hypodermic needle, into which was inserted an insulated copper-constantan couple. The cold junctions were kept at constant temperature in a thermos flask, and the thermoelectric currents were measured with a mirror moving-coil galvanometer. The whole was calibrated by placing the junctions in water at various temperatures.

Fig. 1 is the record of an experiment in which the temperatures of the rectum and of the inside of the heart were taken simultaneously, while the marmot was being awakened by heat.

The marmot was placed in a large glass desiccator; this in turn stood in water which was warmed with an electric heater. The temperatures of the air inside the desiccator and that of the water were observed. The rectal temperature was observed with a thermopile, the point of which was inserted to a distance of about 2 cm. up the rectum. The heart temperature was observed with the "needle" already described. The whole experiment took about eight hours.

The temperatures of the heart and rectum were at first identical (to within two-tenths of a degree); but soon, as the external temperature rose, that of the rectum became higher than that of the heart, and remained about 2 to 3 degrees higher until the heart temperature was 14.4° C., i.e., about

five and a-half hours from the commencement of the experiment. After that point the marmot woke, and the heart temperature rose rapidly ; while it was

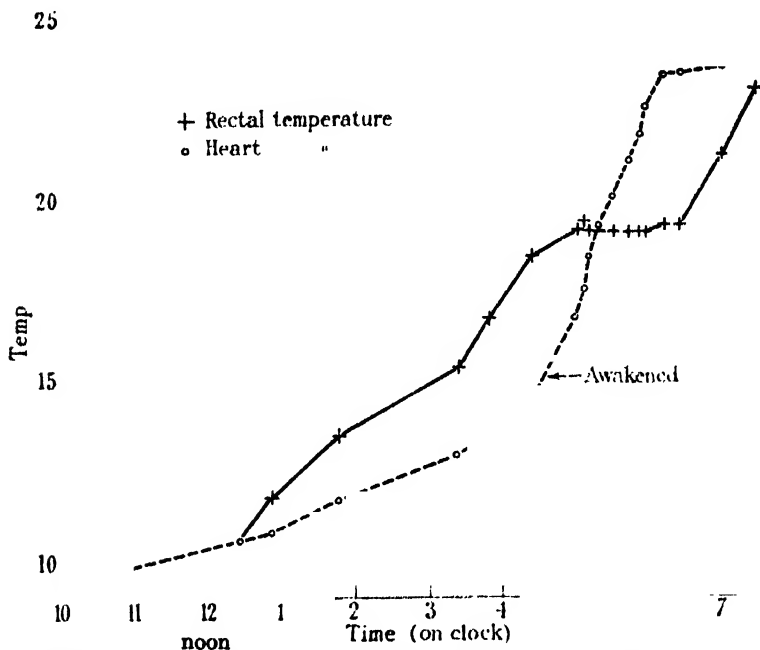


FIG. 1.—Rectal and Heart Temperatures of Marmot. Ordinate == temperature °C. Abscissa == Time of Day.

doing so the rectal temperature remained almost constant, so that, in about half an hour, the heart temperature became equal to the rectal temperature. In another hour the former exceeded the latter by over 4 deg.

When the marmot is asleep, and the room temperature not altering rapidly, the rectal temperature may be taken as a rough index of the body temperature, to within about 4° C. Quincke found differences up to 3 deg. This we have confirmed, but only in those circumstances.

		Quincke, 1882.	Snyder, 1908.	Buchanan, 1911.
Temperature °C.—				
Heart	..	12.4	10	10.1
Rectum	..	10.8	9.8	13.7

#### *Heart Rate.*

Two experiments, one of which was successful, were performed on the perfused hearts of marmots, guillotined during hibernation. In these, the

temperature was correlated with the rate of the heart beat and the electrocardiogram. There are no previous records, so far as we know, on the perfused hearts of hibernating animals.

It was only possible to perfuse the heart between the temperatures of 15.3 and 28.3° C. At the lower temperature the heart seemed in danger of stopping, and at the higher temperature a 3 : 2 block appeared, and quite soon the heart stopped. It had then been perfused for three hours.

Table I gives the data obtained in this experiment.

Table I.

*Experiment No. 6. 3/4/1930. Perfusion of the isolated heart of the Marmot.*

No.	Time.	Thermometer.	Temperature.	Heart rate.	P-R Interval.
2	12.45 p.m.	28° C.	28° C.	84	0.13 secs.
3	1.4	26	26	63	0.14
4	1.19	24.5	24.5	58	0.15
5	1.27	23	23	56	0.18
6	1.37	21.5	21.5	50	0.195
7	1.44	20.0	20.0	44	0.21
8	1.55	18.5	18.5	36	0.165
9	2.4	17.0	17.0	16.5	0.165
Nodal rhythm					
10	2.15	16.0	16.0	5.8	—
11	2.35	15.0	15.3	5.7	—
12	2.47	17.5	17.5	6.4	—
Sinus rhythm returned at 23.5					
13	3.00	23.0	22.7	20	0.23
14	3.10	25.0	24.0	51	0.19
15	3.15	26.5	25.2	52	0.155
16	3.34	28.0	26.4	54	0.15
17	3.45	30.0	28.0	57	0.15
18	3.55	31.0	28.3	56	0.19
				3 : 2 block	

At the end of the experiment the flow of perfusion fluid became very small so that the temperature registered by the thermometer differed widely from that of the heart-muscle. The muscle temperature was therefore checked by means of a needle thermopile. Temperatures in column 4 are the corrected temperatures obtained in this way.

The records were taken with the string galvanometer. The heart was perfused through the coronary arteries by means of a cannula tied into the aorta. The cannula carried a thermometer reading to 0.5° C., the bulb of the thermometer being placed as close to the heart as possible. The perfusion

pressure was about 1 m. of saline and was maintained by a Mariotte's bottle suspended at a suitable height. From the Mariotte's bottle the saline passed to a glass tower where oxygen was constantly bubbled through it, and thence to a glass coil immersed in a water bath which could be maintained at any desired temperature. From the coil the solution passed to the cannula, entering by a side tube. The perfusion was started at room temperature ( $19^{\circ}\text{C}.$ ) and the temperature of the water bath was gradually raised until the thermometer in the cannula registered  $28^{\circ}\text{C}.$  The temperature of the bath was then gradually lowered by the addition of small pieces of ice, records of the electrocardiogram being taken at every  $1.5^{\circ}\text{deg}.$  The lowest temperature recorded in the cannula was  $15^{\circ}\text{C}.$  Finally the temperature was raised again to  $31^{\circ}\text{C}.$  by warming the water bath.

The composition of the Ringer's fluid was as follows: —NaCl 0.85 per cent.,  $\text{Na}_2\text{HPO}_4$  0.06 per cent., KCl 0.042 per cent., Dextrose 0.1 per cent.,  $\text{CaCl}_2$  0.024 per cent. The  $p_{\text{H}}$  was adjusted to 7.5 by addition of HCl and comparison by indicator with a standard buffer solution. In general terms, within the limits of temperature stated, the heart rate rises with the temperature, but the most interesting relation between the two is exhibited when the logarithm of the heart rate is plotted against the reciprocal of the absolute temperature. It then appears, that in the region of 16 to  $18^{\circ}\text{C}.$  there is an abrupt, almost vertical, rise, and that after  $18^{\circ}\text{C}.$  the points lie practically on a straight line

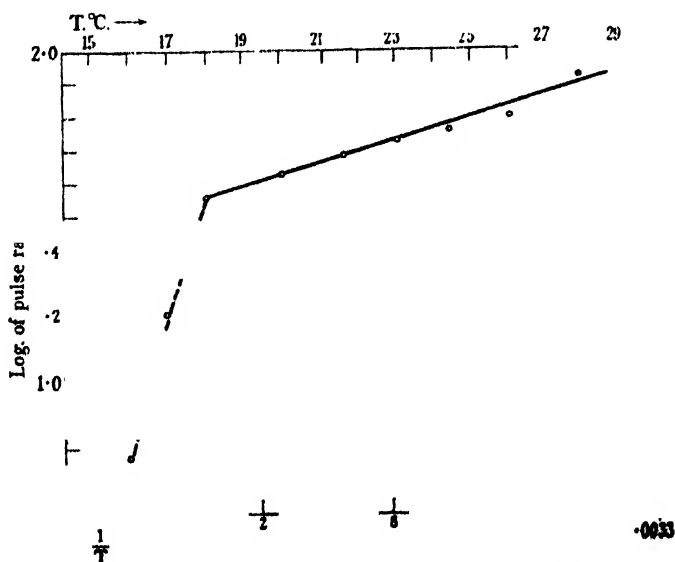


FIG. 2.—Excised Heart of Marmot. Logarithm of heart-rate plotted against reciprocal of absolute temperature.

having a much gentler inclination, which corresponds to a temperature coefficient of 2.1 for the process. This agrees with the figure found by Langendorff for the isolated cat's heart.

Above the temperature of 17° C. nothing recorded on the electrocardiograms deserves comment. Below that temperature the shape of the curve changes, and strongly suggests the substitution for the normal rhythm of a nodal one. This alteration took place at the same temperature as the marked decrease in rate.

#### *The Heart Rate in the Intact Animal.*

The rate and to some extent the character of the pulse in intact hibernating animals have already formed the subject of several researches.

Miss Buchanan (1913) noted that the number of heart beats per minute rose from 76 at 10° C. to 600-900 at 40° in bats, and made similar observations in hedgehogs. Hecht (1915) in the marmot noted a rise in rate from 38-43 at 11° C. to 160-206 at 38°, while Tait (1922) in the woodchuck found a heart-rate of 2 at 0° and of over 120 at 40°.

Miss Buchanan used the capillary electrometer, and observed not only the change in rate, but also, at low temperatures, complete dissociation of the auricles and ventricles (Buchanan, 1913).

Five experiments were carried out successfully by us, in which the temperature and heart beat were recorded during the period of waking. Great pains were taken to ensure that the marmots were not only cold, but asleep. They were left undisturbed in the desiccator for several days before the experiment, with the leads from the electrocardiogram in position, and with iced water surrounding the desiccator. The whole apparatus surrounding the marmot was in a glass chamber which could be closed. The leads were taken to a Matthews Oscillograph (1928), two floors below, and there also was placed the galvanometer, connected by wires to the thermocouples in the marmot.

We began each experiment with the animal cold, and warmed it up. Experiment 4 may be taken as typical, and the results obtained from it will be amplified by references to some of the others.

From 10° C. to about 14° C., the heart rate rose gradually with the temperature in a normal way, but within the next three degrees there was a great increase in rapidity, namely, from 7.9 to 85.7 per minute; 14.5° C. was the temperature at which the marmot first made the sort of movements which convey the impression of becoming awake. We use the word "awake" with some caution, because complete wakefulness might be held to imply the com-

plate transition from the hibernating to the summer conditions, including deep seated alterations in the glands, etc. We have no reason to suppose

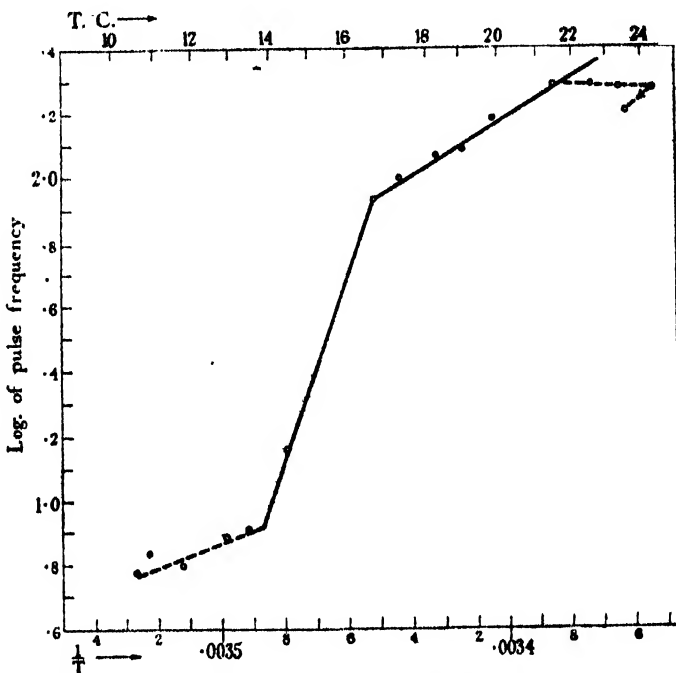
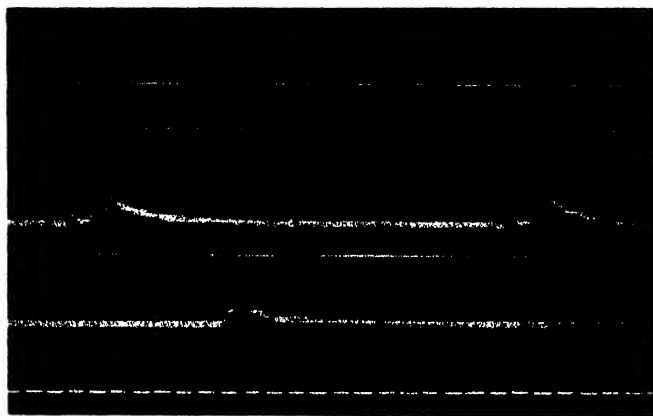
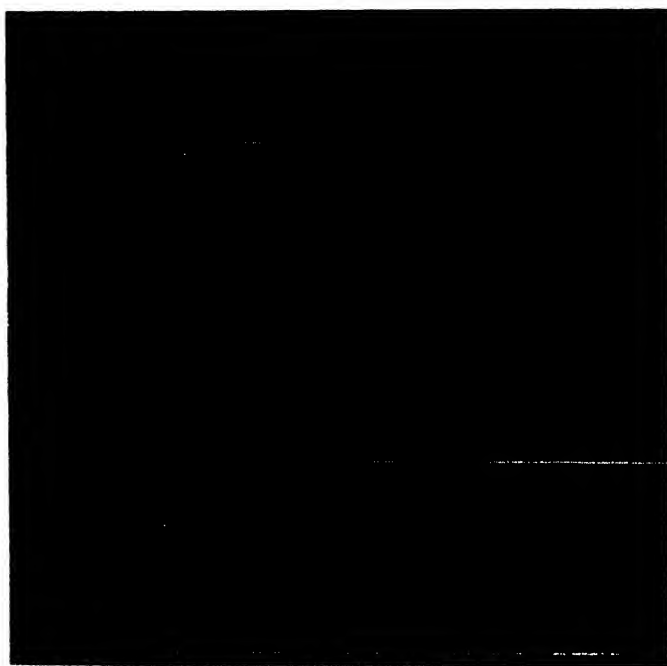


FIG. 4.—Logarithm of pulse frequency plotted against reciprocal of absolute heart temperature in Experiment 4.

that this took place. The foot-movements and poise which are associated with wakefulness could easily be seen through the glass; if cooled down again the animal returned to the condition of obvious hibernation, though sometimes it took a day or two for sleep to become profound. Between 17 and 22 degrees the heart rate again rose gradually with the temperature, and above 22° C. another set of irregularities appeared, which were followed further in the next experiment. Experiment 5 shows the same general relationships up to 20° C. or a little over, but at the next stage there is a great drop in the average heart rate. The irregularities recorded at that point in Experiment 4 were under suspicion, as the thermocouple was observed to have come out of the heart-tissue shortly afterwards, and it appeared to be in the pericardial cavity; but in Experiment 5 there is no doubt about their reality, and at 29° C. they developed into a definite arrhythmia. The arrhythmia was very pronounced, and was of the type generally associated with the action of the vagus. We have no positive evidence of vagus



**FIG. 3.**—Electrocardiogram at 25.2, 17 and 16 °C, respectively. Read from right to left. Time marker,  $\frac{1}{2}$  second.



**FIG. 5, a-d.** Four consecutive pairs of heart beats, which take place at quarter-minute intervals. Time marker, 1 second.



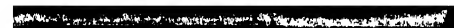


FIG. 6.— See text. Time marker, 1 second.



FIG. 7. *a* and *b*. Electrocardiograms at 23° C. and 26° C. respectively, the former shows no arrhythmia, the latter shows commencing arrhythmia. Time marker, 1 second.



FIG. 8.—The upper and lower portions read continuously. Same experiment as Fig. 29.10° C. Arrhythmia well established. Time marker, 1 second.

action at temperatures lower than this. The data of Experiment 5 are given below.

Table II.  
*Results of Experiment 5.*

No.	Time.	Rect. Temperature.	Heart Temperature.	Heart Rate (beats per minute).	P.Q.
		°C.	°C.		
502	12.35	9.8	12.6	93	0.20
503	12.40	9.8	16.0	115	—
504	12.53	9.4	17.7	160	0.16
505	1.00	9.8	20.2	183	—
506	1.17	9.4	23.7	190	0.091
507	1.23	8.8	23.8	165	0.10
508	1.25	8.8	24.8	160	0.088
509	1.28	8.8	26.0	165	0.106
510	1.38	8.8	27.2	163	0.091
511	1.45	8.8	28.0	200	0.103
512	1.56	8.1	29.1	Max. 171 Min. 107 Mean 118	Max. 0.085 Min. 0.075
513	2.6	8.1	29.4	220	0.095
514	2.10	8.1	29.4	Max. 197 Min. 55 Mean 137	Max. 0.097 Min. 0.076
515	2.16	8.1	29.7	110	—
516	2.25	8.8	30.1	Max. 210 Min. 110 Mean 202	Max. 0.10 Min. —
517	3.05	9.8	30.5	—	—
518	3.30	11.4	30.2	Max. 160 Min. 53 Mean 98	Max. 0.094 Min. 0.063
523	6.07	16.8	29.2	Max. 180 Min. 72 Mean 130	Max. 0.078 Min. 0.067

The first great crisis, that which takes place at 14 to 17° C. in the living marmot, is not easy to explain. At first we were inclined to attribute it to direct or indirect nervous influences, such as direct sympathetic action or adrenaline secretion; but it closely resembles the effect seen at nearly the same temperature in the excised heart, and that was clearly associated with the abolition of the function of the sinus as pace-maker. It is difficult from our tracings of Experiment 4 or 5, to decide whether the sinus was or was not the pace-maker below 14° C.

Apart from the slowness of the rhythm at low temperatures, irregularities appear, for instance in Experiment 2. The heart beats fell into pairs with long intervals; two beats separated by about three seconds took place about

every quarter of a minute. The first beat was apparently normal in type, but the second always differed from the first, in the way seen in fig. 5 (Plate 17). This figure shows four consecutive pairs of beats, *a*, *b*, *c* and *d*, assuming that  $c_2$  is a beat. With regard to  $c_2$ , it should be stated that there is no undulation at all of this size in the parts of the record which are omitted. There is not sufficient evidence on which to decide whether the apparent absence of beats in the pauses was due to a complete suspension of all rhythmic phenomena in the heart, or was due to a block. Records such as these raise the question as to whether a nodal beat appears in the living marmot as it does in the excised heart; on this we prefer to suspend judgment. Thus the tracings in fig. 6 (Plate 18) are taken from the same experiment as those in fig. 5 (Plate 17), but at  $8.9^{\circ}\text{C}$ . instead of  $9.6^{\circ}\text{C}$ . The *p*-waves are obviously less conspicuous, if, indeed, they exist.

As regards the general features in the rhythm and character of the beat at higher temperatures, fig. 7, *a* (Plate 18), shows a record taken at  $23.7^{\circ}\text{C}$ .; this shows no arrhythmia, whilst at a slightly higher temperature,  $26^{\circ}\text{C}$ . (fig. 7, *b*), there is evidence of commencing arrhythmia. This becomes more marked and is well shown in fig. 8 (Plate 18), the upper and lower portions of which are continuous. Here the most rapid phase of the rhythm was over three times as fast as the slowest. The P—R interval is shorter at the slower rate. In general, however, a rise of heart temperature is associated with a shortening of all phases of the cardiac cycle, *e.g.*, the P—R interval and the ventricular complex.

We would like to express our thanks to the Rockefeller Foundation for a grant given to one of us (Endres) for research on hibernation, with which were also defrayed the expenses of obtaining the marmots.

#### REFERENCES.

- Buchanan, 1911. 'J. Physiol.,' vol. 42, p. xii.  
Buchanan, 1913. 'Proc. XI<sup>e</sup> Congrès Internat. Physiol., Groningne.'  
Hecht, 1915. 'Z. Ges. Exp. Med.,' vol. 4, p. 259.  
Matthews, 1928. 'J. Physiol.,' vol. 65, p. 226.  
Quincke, 1882. 'Archiv Exp. Pathol. Pharmacol.,' vol. 15, p. 1.  
Snyder, 1908. 'Amer. J. Physiol.,' vol. 22, p. 330.  
Tait, 1922. 'Amer. J. Physiol.,' vol. 59, p. 467.

*Observations on Certain Physiological Processes of the Marmot.*  
 II.—*The Respiration.*

By G. ENDRES and H. TAYLOR, Physiological Laboratory, Cambridge.

(Communicated by J. Barcroft, F.R.S.—Received July 8, 1930.)

Many observers, of whom the earliest appear to have been Valentin (1870), Dubois (1896), Patrizi (1897) and Pembrey and Pitts (1894), have made graphic records of the respiratory movements in marmots. These were all registered by stethographic and similar methods. Such do not give quantitative data of the depth of respiration and of the total ventilation. The depth, the rate, and incidentally the total ventilation have now been studied simultaneously by an apparatus described by one of us (Endres, 1930). These records were taken either when the marmot was asleep in cold air with which it was in equilibrium, or when it had just been removed from such an atmosphere to a warmer one. In the latter case, the temperature of the marmot rose and ultimately the animal awoke. It is known that, when the marmot is awaking, its rectal temperature frequently differs from that of its mouth or its heart (see previous paper); and that when the marmot is asleep the disparity is less. Nothing, so far as we know, is known about the temperature of the marmot's brain (respiratory centre) as compared with other parts of the body. It cannot be claimed that even during sleep the rectal temperature is more than an approximation, to within a few degrees, of the body temperature.

The general features shown by the respiratory records observed when a marmot was asleep during hibernation are as follows. Taking Experiment 7 as an example, the rectal temperature of the marmot was  $1^{\circ}\text{C}$ . at the beginning of the experiment. Though the marmot was asleep its respiratory activity at this temperature was relatively high: in this connection it must be remembered that sufficient cold wakes the marmot. It has been suggested to us by Pembrey that, on the border of awakening, the contact between the nose and the mask may have stimulated respiration.

Figs. 1, *a*, and 1, *b*, show typical tracings with the spirometer when the rectal temperature was  $1.2^{\circ}\text{C}$ . and  $3.2^{\circ}\text{C}$ . respectively. The former was taken at about nine in the morning and the latter late the same afternoon. In the latter tracing the respirations were, on the average of several minutes,

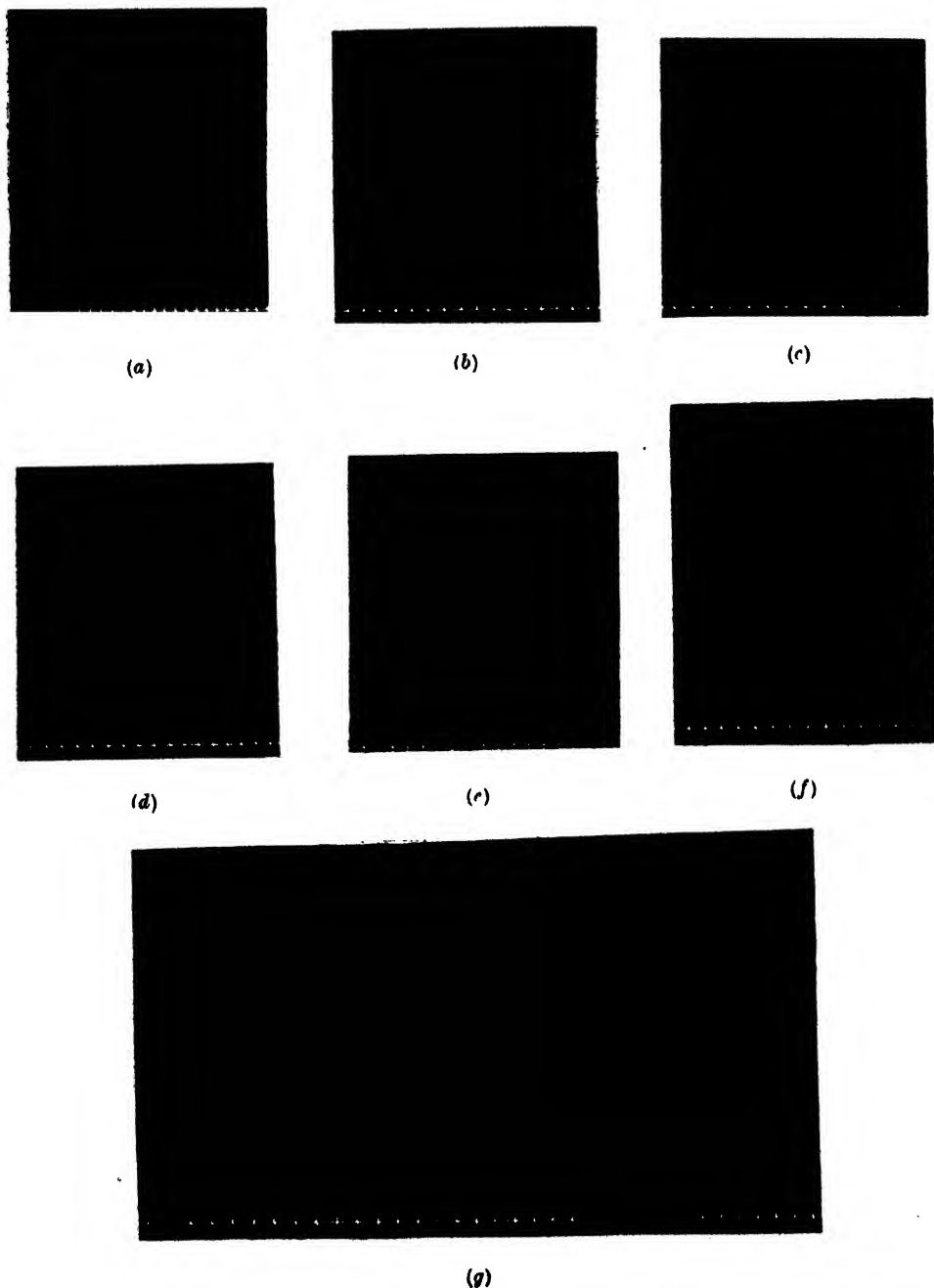


FIG. 1.—Tracings of the volume of air inspired by the marmot. A rise of the lever denotes inspiration. The marmot was in an air-tight box, fitted with a spirometer, the movements of the lever of which were recorded. It breathed (through valves) air from outside the box. Time,  $\frac{1}{2}$  minutes.  $a = 1.2^{\circ}\text{C.}$ ;  $b = 3.2^{\circ}\text{C.}$ ;  $c = 6.2^{\circ}\text{C.}$ ;  $d = 11^{\circ}\text{C.}$ ;  $e = 16.9^{\circ}\text{C.}$ ;  $f = 17^{\circ}\text{C.}$ ;  $g = 20^{\circ}\text{C.}$  (rectal temperatures).

slightly shallower and slower than the former; the total ventilation was slightly less at the higher rectal temperature. The difference in the rectal temperatures of the animal when these tracings were taken was so small that we cannot argue any known difference in the temperature of the brain, *i.e.*, in the respiratory centre or centres. On the following morning, however, tracings were taken commencing at 5° C.—a good example is shown in fig. 1, *c*. Both the rate and the depth were markedly less than in fig. 1, *a*, and therefore the total ventilation is considerably reduced; in fact, it was about half the value of that on the day previously.

From this point as the animal was warmed up the rate and depth of

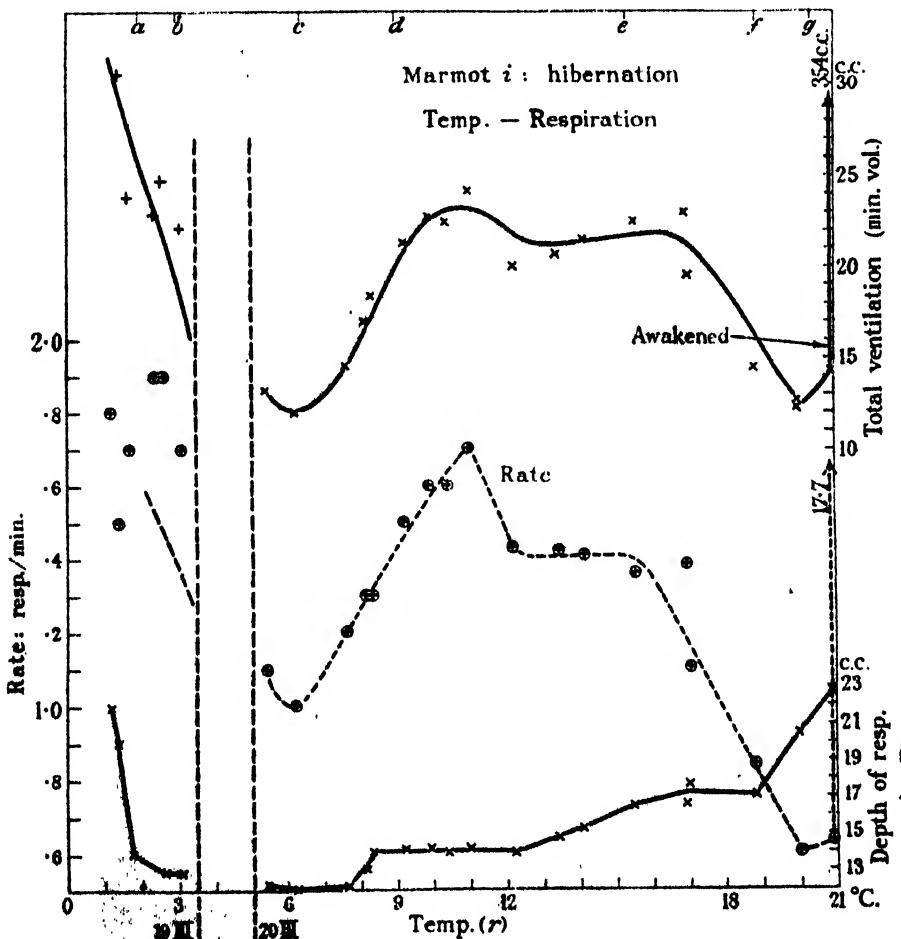


FIG. 2.—Experiment 7. Total ventilation rate of respiration and depth of respiration plotted against rectal temperature; *a-g* correspond to the tracings labelled with the same letters in fig. 1.

respiration rose, the depth more markedly than the rate, so that the curve of the total ventilation reached a plateau value—figs. 1 and 2, *d* and *e*—from which it descended at a higher temperature. When, therefore, the rectal temperature was 20° C. (figs. 1 and 2, *g*), the total ventilation was about the same as when it was 6° C.

This falling off in the rate of respiration, even though accompanied by a slight rise in the depth is a very striking phenomenon. The main features of Experiment 7 were reproduced in Experiment 8.

Fig. 3 shows the respiratory tracing when the marmot was awakening.

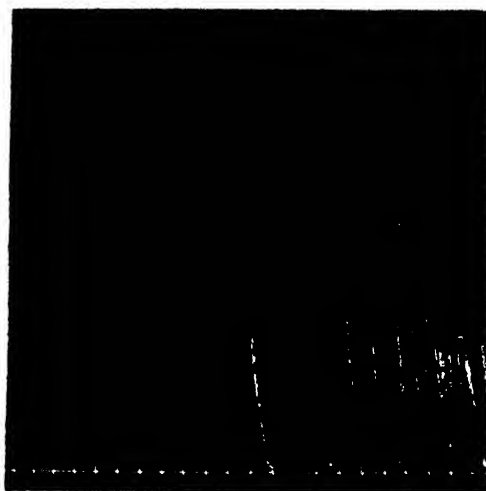


FIG. 3.—Portion of the same record as fig. 1, showing the alteration in respiration when the marmot awakes.

We may pass to the consideration of the form and time relations of the curves of single respirations, taken on a more rapidly moving surface that was used for fig. 1.

Fig. 4 shows records of three individual respirations taken at different temperatures, viz., 4·8° C., 8·1° C. and 19·6° C., all with the marmot asleep in hibernation. The curves shown are not the same as the actual tracings. In the actual tracings, had the drum been stationary, a movement of the lever would have registered an arc on the paper. The distances of the various parts of the curve from such an arc have been measured and laid off on squared paper from a vertical ordinate, so that the distance of the curve from the ordinate at any point gives a correct time measurement.

The slowest rate of respiration which we observed was that of about one in

every five minutes. At the time when tracing, shown in fig. 4, *a*, was taken, the respirations took place, on the average, at approximately three-minute

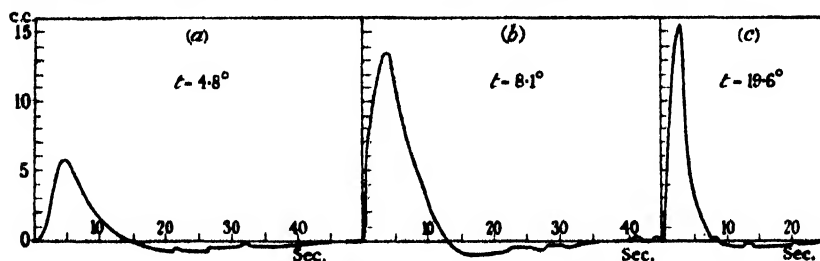


FIG. 4.—Records of individual tracings, analysed so that the tracing refers to a vertical ordinate. Rectal temperatures, *a* = 4.8° C., *b* = 8.1° C., and *c* = 19.6° C. respectively. The tracing in each case ends when the respiration appears to be complete and does not include the pause which separates it from the subsequent respirations.

intervals; the tracing shows the first 45 seconds which is the time from the commencement of inspiration till the chest, after expiration, returns to its initial volume.

Rectal Temperature	4.8° C.	8.1° C.	19.6° C.
A.—Volume inspired	5.6 c.c.	13.8 c.c.	15.4 c.c.
B.—Time of inspiration (seconds)*	4.5	3.5	3.0
C.—From height of inspiration to base-line (seconds)	10.0	9.5	5.5
D.—From base-line to deepest point of expiration (seconds)	13.5	6.5	6.7
E.—Time of expiration (C + D above) (seconds)	23.5	16.0	12.2
F.—Time from deepest point of respiration to return of chest to initial volume	22	19	12.5
G.—Total time of one respiration (seconds)	45	35	25
H.—Pause (seconds, approximate)	180	60	54
Rate of respiration per minute	0.33	1	1.1

\* In these measurements, the curves have been "faired" to eliminate the small notches due to heart beats.

It is to be noted in view of what will be said later, that the inspiration period shortened as the temperature rose. Expiration is a very definite muscular movement and produces a contraction of the chest to a smaller volume than either that at the commencement of inspiration or during the pause between respirations. Such curves as shown in fig. 1 might give the impression that the descent of the lever below the base line was due to an overthrow, but this was not the case. It must be remembered that the whole process of respiration is very slow, the time intervals on the tracing being half minutes. Moreover, a presentable imitation (in time and form) of such a curve as shown in fig. 4, may be made by forcing air into and drawing it out of the chest with a syringe attached to the recording spirometer; such a curve never shows any inertia effects.



*The Effect of Carbon Dioxide.*

The effect of carbon dioxide was observed in the following way. The animal was placed in the desiccator and the respiratory data measured as has been described. The carbon dioxide was administered by a method already described by one of us (Endres, 1927), in detail. Four spirometers of thin celluloid sheeting were filled with the gas mixtures of air containing known quantities of carbon dioxide; the capacity of each spirometer was about four litres. These, one after the other, were attached to the inspiratory tube of the valves attached to the mask. In the case of animals after hibernation the animal was placed in a special box (Endres, 1930).

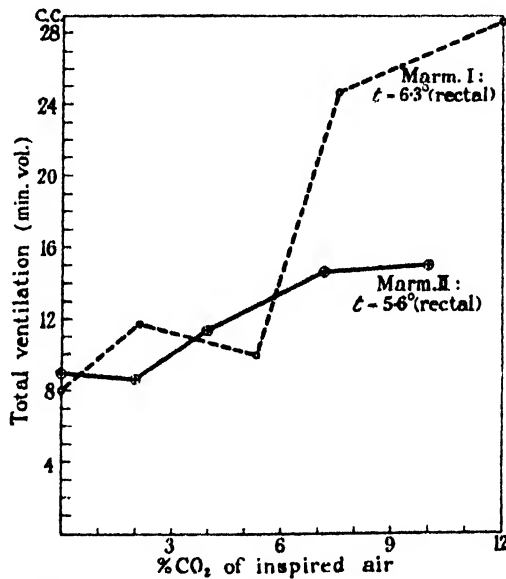


FIG. 5.—The effect of carbon dioxide on the total ventilation of the hibernating marmot.

The total ventilation was at most 28 c.c. a minute in the hibernating animal, with 12 per cent. carbon dioxide (fig. 5), but it rose to about 1600 c.c. in the animal awake after 6.1 per cent. carbon dioxide.

The following tables give an idea of the data obtained, and in spite of a certain amount of irregularity in the respiration, the following facts appear to be established.

## Hibernating Marmot (25.11.29). Weight, 2.13 kilos.

Temp. (Rect.).	CO <sub>2</sub> - content of inspired air.	Respiration Rate.		Period of observ- ance.	Depth of Respiration.		Total Ventilation.	
		Average rate per minute.	Maximum and Minimum time for Respiration (minutes).		Average (c.c.).	Extremes (c.c.).	c.c.	Increase above initial value (c.c.).
5.6°	per cent.			Min.				
5.6°	—	0.68	(3.2-0.8)	66	13.1	(11.3-14.8)	8.99	—
5.6°	2.0	0.61	(3.3-0.6)	75	14.3	(12.5-15.6)	8.72	(-0.27)
5.6°	4.0	0.75	(4.3-1.0)	40	15.0	(13.8-16.0)	11.25	2.26
5.6°	7.1	0.95	(2.3-0.7)	41	15.5	(14.2-16.8)	14.7	5.71
5.6° (6.2° mouth)	10.0	0.96	(2.2-0.7)	50	15.6	(13.8-16.8)	15.0	6.01

## Marmot, normally awake (28.5.30.)

37.8°	—	8.0	—	—	21.8	—	174	
	1.5	8.6	—	—	37	—	318	144
	2.9	9.0	—	—	52	—	468	294
	6.1	25.5	—	—	63	—	1606	1432

Carbon dioxide produces little increase in either the rate, the depth, or the minute volume of respiration, unless the percentage in the inspired air exceeds five. So far as rate is concerned, this is true of both the hibernating and the normal "summer" animals. In one of the two hibernating marmots studied, carbon dioxide produced a sudden augmentation in respiration when the percentage reached five and a half. In the other marmot the effect was less. During hibernation, however, even 12½ per cent. of carbon dioxide only produced a three-fold increase in the total ventilation. The effect of carbon dioxide on the marmot in the "summer," i.e., the homoiothermic condition is quite different and the results obtained by us are very like those obtained by Endres on the rabbit. Fig. 6 shows the increase in the total ventilation (in cubic centimetres per minute), caused by carbon dioxide in the case of (a) two rabbits; (b) two hibernating marmots; and (c) one "summer" marmot.

In the case of the "summer" marmot as in man (Haldane and Priestley, 1905) and other mammals (Scott, 1908), moderate increase in the carbon dioxide in the inspired air produces almost no effect on the rate of respiration, the obvious effect being in the depth which increased progressively with an increase of carbon dioxide in the air breathed. One of the most interesting

## G. Endres and H. Taylor.

effects of carbon dioxide, however, affected the shape of the respiratory curve, which, on account of the slowness of the whole process, is particularly easy to observe.

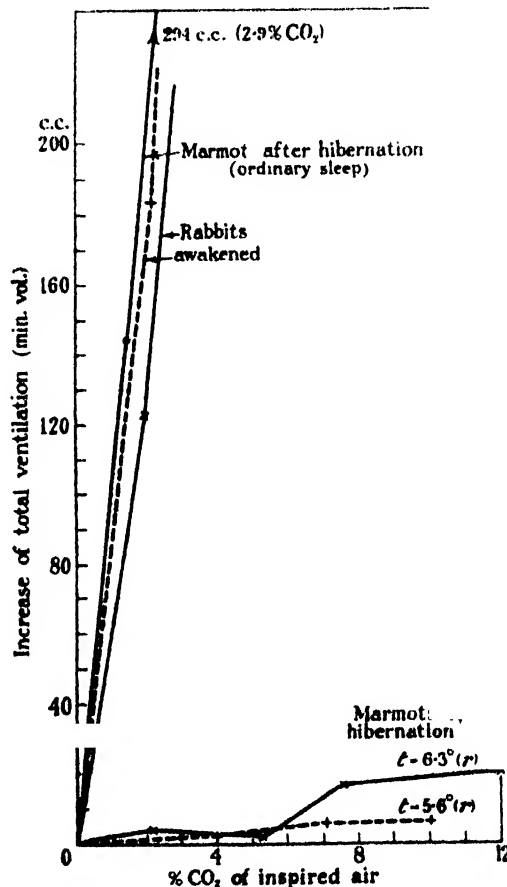


FIG. 6.—Percentage increase in total ventilation of two marmots asleep and hibernating. One marmot asleep but not hibernating and two rabbits awake.

Fig. 7 (A and B) shows two curves with the animal breathing (A) ordinary air, and (B) air containing 10 per cent. of carbon dioxide respectively. The inspiration commences at the same rate in each case and it is only when the inspiratory effort approaches completion that the curve diverges. In the marmot breathing 10 per cent. carbon dioxide, inspiration continues unchecked for a longer period, so that the summit of inspiration is later by about a second. If, therefore, we say that in curve B, respiration is "stimulated" by carbon dioxide, as compared with Curve A, we use the word "stimulate" in rather a special sense; in reality we mean that the change of phase from inspiration to

expiration is postponed and that inspiration proceeds till the change comes. When expiration does set in, however, it seems to set in with greater force in

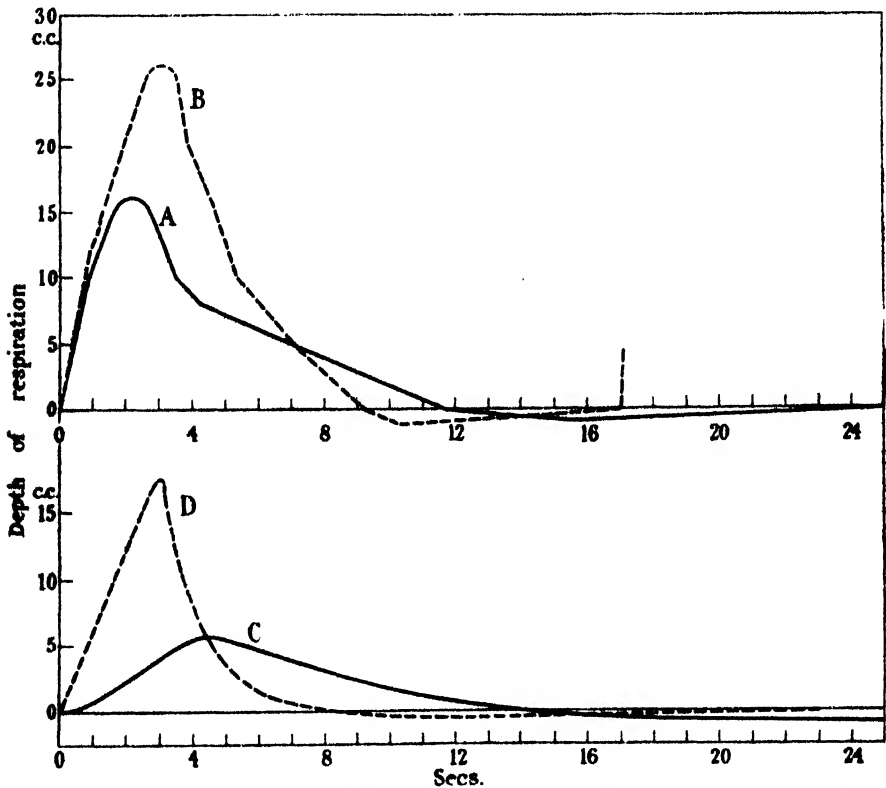


FIG. 7.—A tracing of the volume of air breathed by hibernating marmots. The animal used for A and B was not the same as that used for C and D. Tracing C reaches the base-line at 45 seconds from the commencement. Temperature of marmot in C,  $4.8^{\circ}$ ; in D,  $19.6^{\circ}$ .

the animal which is breathing carbon dioxide, the whole time occupied by the expiratory phase of a respiration being shortened.

The effect of carbon dioxide is in marked contrast to the effect of raising the temperature, as shown in fig. 7, C and D. At the warmer temperature every phase of respiration from the very commencement is quickened.

This conception of the effect of carbon dioxide is somewhat different from that put forward by Barcroft (1919), who suggested that carbon dioxide made both the expiratory and the inspiratory phases of this curve more steep as well as increasing its amplitude. Here the expiratory phase is more steep, but not the inspiratory phase. Till further investigations are carried out-

such curves as are shown in fig. 7, A and B, must not be regarded as necessarily true of mammals generally.

*Periodic Breathing.*

No such complete example of periodic breathing has been observed by us as that figured by Pembrey (1901) in which periods of waxing and waning are separated by periods of practically complete apnoea. Fig. 8 is typical of what we have occasionally seen, but even this is rare.



FIG. 8.—Tracing of respiration approaching the periodic type.

REFERENCES.

- Barcroft (1919). 'J. Physiol.,' vol. 53, p. lxviii.  
 Endres (1930). 'J. Physiol,' vol. 70, *in the press*.  
 Endres (1927). 'Z. Biol.,' vol. 86, p. 147.  
 Dubois (1896). 'Physiologie comparée de la marmotte.' Paris.  
 Haldane and Priestley (1905). 'J. Physiol.,' vol. 32, p. 225.  
 Patrizi (1897). 'Z. Physiol.,' vol. 11, p. 567.  
 Pembrey (1901). 'J. Physiol.,' vol. 27, p. 78.  
 Pembrey and Pitts (1899). 'J. Physiol.,' vol. 24, p. 305.  
 Scott (1908). 'J. Physiol.,' vol. 38, p. 301.  
 Valentin (1870). "Untersuchungen zur Naturlehre des Menschen und der Thiere von Moleschote," vol. 10, p. 590.

*Observations on Certain Physiological Processes of the Marmot—  
III, IV and V.*

By G. ENDRES, Physiological Laboratory, Cambridge.

(Communicated by J. Barcroft, F.R.S.—Received July 11, 1930.)

*III.—The Oxygen Saturation of the Arterial Blood.*

The oxygen saturation of the arterial blood was the subject of observations upon two hibernating marmots. Blood from the left ventricle was withdrawn by the method of cardiac puncture. The blood was not exposed to air. Post-mortem examination of the heart wall established the facts that the needle penetrated the wall of the left chamber and not that of the right. The analysis of the blood was made by the differential method. The marmots were sleeping soundly.

Date.	Rectal Temperature.	Saturation (per cent.).
25.3.30	9.2° C.	96.4
25.3.30	11.4° C.	95
		93.5
		95.2

*The effect of Alterations of Temperature on the Oxygen Equilibrium of the Red Corpuscles.*

Experiments were made on the relation of temperature to the oxygen-combining power of the corpuscles; I am grateful to Dr. Stier and Dr. Rothschild for assistance in these experiments.

The two experiments so made differed in that the first was carried out at constant pressure of carbon dioxide and the second at constant, or approximately constant,  $p_{\text{H}}$ . The first series therefore is comparable with the work of Brown and Hill (1923) on the effect of temperature on the oxygen dissociation curve of human blood. In their research the blood was equilibrated with oxygen in the presence of 40 mm.  $\text{CO}_2$  pressure throughout. The experiments at constant  $p_{\text{H}}$  are more comparable with those of Barcroft and King (1909), which were carried out in the absence of carbon dioxide and with the kinetic determinations of Hartridge and Roughton (1923). Brown and Hill obtained

a temperature coefficient of about 1.7 when the pressure at which the hæmoglobin was 50 per cent. saturated with oxygen, was plotted against the reciprocal of the absolute temperature. Barcroft and King obtained a higher temperature coefficient for the blood of herbivores (about 3.4) as did Hartridge and Roughton for the blood of sheep (2.7 — 4).

In the second experiment here described the corpuscles were washed three times in Ringer's solution and suspended in a sample of that solution which was buffered to  $p_H$  7.76.

The temperature coefficients determined at constant pressure of carbon dioxide and at constant  $p_H$  may be expected to differ. At a given pressure of carbon dioxide the blood is likely to become more alkaline as the temperature rises. This is because the carbonic acid in solution diminishes more than the chemically combined carbonic acid and therefore the ratio of dissolved to combined carbon dioxide changes. Again the effects of temperature on the degree of ionisation of the relevant substances and on the Henriques effect (Henriques, 1928), if that is admitted, are uncertain factors.

Determinations were made in the first experiment at 8°, 17°, 25°, 32° and 35° C. The object in each case was to arrive at the oxygen pressure at which the hæmoglobin in the corpuscles was 50 per cent. saturated. The actual procedure was, at each temperature, to determine two points one a little above 50 per cent. saturation and the other a little below, and to arrive at the 50 per cent. point by a process of interpolation. This interpolation can be made with considerable accuracy by the use of Hill's equation. Indeed, if only one point is known, and that not too remote from the 50 per cent. saturation point, the latter can be calculated with sufficient accuracy. No adherence to the theoretical implications of Hill's equation is involved, the equation is merely used to give correct numerical approximations. For that purpose, it is extremely valuable; at the middle of the dissociation curve, lines deduced from it are not appreciably in error.

The apparatus used was that form of the differential manometer described for the estimation of 0.1 c.c. of blood. No ferricyanide was employed, since it has been shown by Parsons and Parsons (1927) and by Litarczek (1928) that the use of ferricyanide may lead to considerable irregularities. Such irregularities have been observed in blood of rodents by Douglas (1910), and others; there was no reason to suppose that the blood of the marmot was free from them. The following observations were made:—

I.—CO<sub>2</sub> Pressure, 40 mm.

Temp. °C.	Pressure of oxygen. mm.	Saturation per cent.	Pressure of oxygen corresponding to 50 per cent. solution.	log P <sub>50</sub> per cent.	$\frac{1}{T_K} \times 10^4$ .
8	5.1	39	4.3	0.64	3559
	3.3 { 34 33 } 33.5		4.5	0.65	
17	14.6 { 79 77 } 78		8.6	0.93	3448
	7.7 { 50 46 } 47		8.6	0.93	
25	14.9	—	—	—	3356
	8 { 31 32 } 31.5		11.2	1.05	
32	21.4 { 61 57 } 59		18.5	1.27	3279
	14.4 { 39 39 } 39		17.0	1.23	
38	24 { 57 47 } 52		23	1.36	3215
7	23.8 { 42 48 } 46		25.7	1.41	

II.—p<sub>H</sub> of Medium in which the Corpuscles were suspended 7.76.

Temp. °C.	Pressure of Oxygen. mm.	Saturation, per cent.	P. at 50 per cent. saturation.	log P <sub>50</sub> per cent.	$\frac{1}{T_K} \times 10^4$ .
9	2.15	78.7	1.37	0.137	3546
15	2.34	58.5	2.0	0.301	3472
21	2.52	46.0	2.6	0.415	3401
27	8.49	71.5	6.2	0.792	3330
33	8.75	52.5	8.25	0.916	3268
39	42.1	43.5	16.0	1.204	3205

The logarithm of the 50 per cent. saturation-pressure is plotted against the reciprocal of the absolute temperature in the Kelvin scale, in fig. 1. With the exception of one point the determinations in the case of each experiment fall as nearly on a straight line as could be expected. In fact, when the complicated chemical background of the union of oxygen with hæmoglobin is



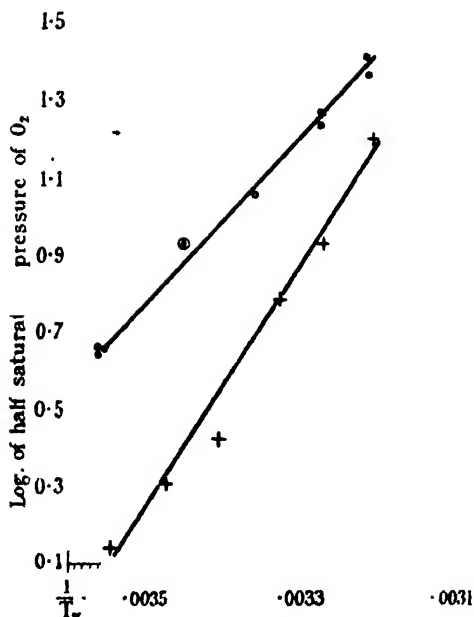


FIG. 1.—Ordinate = log. of pressure at which the hæmoglobin in the corpuscles is 50 per cent. saturated with oxygen. Abscissa reciprocal of absolute temperature. Upper line = corpuscles exposed to 40 mm.  $\text{CO}_2$  pressure. Lower line =  $p_{\text{H}} 7.76$ .

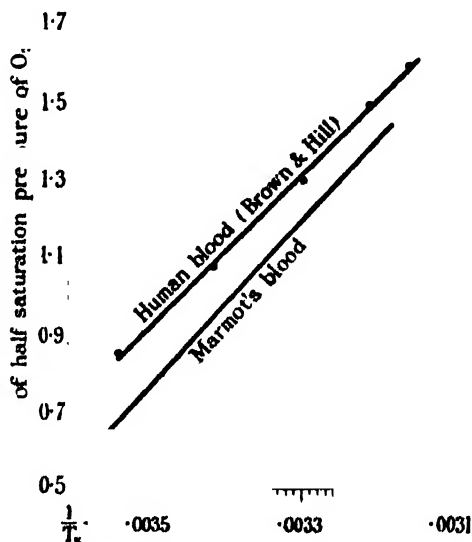


FIG. 2.—Ordinate and abscissa as in fig. 1. Upper line = human blood calculated from Brown and Hill (1923), fig. 4. The point corresponding to Brown and Hill's curve at  $0^\circ \text{C}$ . has been omitted because the half saturation pressure cannot be read with sufficient accuracy from their figure.  $\text{CO}_2 = 40 \text{ mm}$ .

considered, there seems to be little reason to assume that the points should fall on a straight line at all. So far, however, as the points for each experiment are on a line they indicate a temperature coefficient of 1·8 for the corpuscles exposed to 40 mm. CO<sub>2</sub> pressure and for those at approximately 7·76 p<sub>H</sub>, a temperature coefficient of 2·7. The temperature coefficients may be calculated from fig. 1, whilst that of Brown and Hill was calculated from fig. 2.

#### IV. --*Blood Sugar.*

The blood sugar of five animals was determined towards the end of March : the animals were still in deep hibernation and were not awakened by the experiment. The blood was withdrawn by cardiac puncture and the sugar was estimated by the method of Hagedorn and Jensen (1925). Determinations were made again in June when the animals were wide awake. The following were the results : -

#### Blood Sugar. During Hibernation.

		Date	Rectal Temperature.	Blood Sugar.
			°C.	per cent.
Marmot	I	1930. March 25	9·2	0·075 0·071
„	II	„ 25	11·4	0·086 0·089
„	III	„ 25	14·8	0·092 0·090
„	IV	„ 28	15·1	0·081 0·087
„	V	„ 28	15·8	0·092 0·096

#### Blood Sugar during summer.

Marmot	June	36·8	0·134 0·138
		38·1	0·97 0·97
		37·6	0·162 0·160

The above three marmots were survivors of the five whose bloods were estimated in March, but the identity of the individual marmots was lost. We are indebted to Dr. C. G. L. Wolf for making the "summer" estimations.

With regard to the results during hibernation, it may be observed that the blood was never singularly deficient in sugar. Two determinations of human blood estimated at the same time by the same method gave respectively 0·102 and 0·101 per cent. If the tissues of the marmot are living on fat during

hibernation (as is indicated by the low respiratory quotients given in the literature), it is clearly *not* because the blood is deficient in sugar. It seems likely, though not definitely proved, that the stored fat is converted into sugar possibly through the glycogen phase, which sugar is used to maintain the blood sugar level.

The animals whose sugar was estimated during hibernation did not struggle at all. The same animals in summer struggled, especially the last on the list—a fact which may account for the high blood sugars recorded in two cases. The marmots were prone to bite and were held with a towel round the neck, they were also gripped by the fore and hind legs. In this position, care was taken that they could breath satisfactorily, nevertheless, a somewhat cyanosed appearance of the tongue was observed.

#### V. *Sensitivity of the Skin and the Depth of Sleep.*

The skin on the end of the nose was stimulated by a hair such as described by von Frey (1922). The hair used was that described by him for the testing of pain, not touch. The criterion used for sensitivity was the small movements of the head or legs of the animals. There never was opening of the eyes.

Though the pressure of the hair necessary to produce movement varies considerably, it is of a different order of magnitude during hibernation from what it is during normal sleep in summer time as the following table shows :—

Exploration of the Sensitivity of the Skin (Depth of Sleep).

		Temperatures (°).	Stimulus Eliciting Response (Strength of von Frey's hair for pain).
marmot 1	February 26	9.8°	20g
	March 12	12.5°	12g
	March 17	16.4°	10g
marmot 2	February 26	10.5°	14g
	March 12	13.1°	16g
	March 17	15.3°	8g
marmot 3	March 17	6.4°	6g
	February 26	11.4°	8g
	March 12	12.7°	8g
marmot 4	March 17	6.8°	18g
	February 26	10.9°	4g
	March 12	12.2°	6g

Exploration of the Sensitivity of the Skin (Depth of Sleep)—continued.

		Temperatures ( <i>r</i> ).	Stimulus Eliciting Response (Strength of von Frey's hair for pain).
After Hibernation : (ordinary sleep).			
Marmot 1	May 29 .....	37·1°	1 $g$
Marmot 2	May 29 .....	37·6°	1 $g$
Marmot 3	May 29 .....	38·2°	1 $g$

We wish to thank Prof. J. Barcroft, F.R.S., for valuable encouragement, collaboration and criticism.

REFERENCES.

- Barcroft and King (1909). 'J. Physiol.,' vol. **39**, p. 374.  
 Brown and Hill (1923). 'Proc. Roy. Soc.,' B, vol. **94**, p. 310.  
 Douglas (1910). 'J. Physiol.,' vol. **39**, p. 453.  
 von Frey (1922). 'Z. Biol.,' vol. **76**, p. 1.  
 Hagedorn and Jensen (1925). 'Biochem. Z.,' vol. **135**, p. 46.  
 Hartridge and Roughton (1923). 'Proc. Roy. Soc.,' A, vol. **104**, p. 395.  
 Henriques (1928). 'Biochem. Z.,' vol. **200**, pp. 1, 5, 10, 18, 22.  
 Litarczek (1928). 'J. Physiol.,' vol. **65**, p. 1.  
 Parsons and Parsons (1927). 'J. Biochem.,' vol. **21**, p. 1194.

*The Accumulation of Electrolytes in Plant Cells—A Suggested  
Mechanism.*

By G. E. BRIGGS, St. John's College, Cambridge.

(Communicated by F. F. Blackman, F.R.S.—Received August 7, 1930).

*Survey of Mechanism already suggested.*

The analysis of the sap of cells of plants such as *Valonia* and *Nitella* has shown that the concentration of some of the ions is much greater in the sap than it is in the surrounding solution in which the cells have grown. Not only is this so for cations such as K but also for anions such as Cl. Höber and Höber (1928) found that the dilution of sea water with isotonic glucose caused practically no change in the potassium content of *Valonia*. Brooks (1929) added isotonic NaCl to sea water to such an extent that the concentration of potassium in the latter was reduced to a half, and yet there was no appreciable change in the composition of the cell sap of *Valonia*.

To explain their results the Höbers postulated the existence of a membrane around the cell sap consisting of a mosaic of two kinds of areas: one positively charged and permeable to anions only and the other negatively charged and permeable to cations only. Brooks postulated a similar type of membrane to explain the accumulation of ions in the sap.

Michaelis and his co-workers (1927) have investigated collodion membranes which are negatively charged and impermeable to anions. The mobilities of cations through such membranes form a similar sequence to the mobilities in free solution, hydrogen ions having the highest mobility, potassium greater than sodium and the bivalent ions of calcium and magnesium being yet slower. The ratio of mobility in the membrane to that in free solution is greater for the faster-moving ions.

Mond and Hoffmann (1928) prepared a membrane impermeable to cations by treating the collodion with rhodamine, so that it was positively charged. The mobility of Cl ions through such a membrane is greater than that of SO<sub>4</sub> ions, but less than that of CNS ions, which have a smaller mobility in free solution than have Cl ions. In all cases, for both cations and anions in their respective membranes, the mobility in the membrane relative to that in free solution is greater the less the salting-out effect of the ion. Greater salting-out effect is supposed to be connected with greater polarisation of the water molecules

around the ion—that is, greater hydration of the ion. So the greater the hydration of the ion the more is it slowed down by the membrane. Hence it appears probable that size of ion is an important factor in deciding the average rate at which it can penetrate such artificial membranes.

Höber and Hoffmann (1928) have prepared and investigated a membrane composed partly of undyed collodion and partly of collodion dyed with rhodamine. Such a membrane behaves as if it was permeable to both anions and cations, provided they are small enough. It is a membrane of this type that is postulated by the Höbers and by Brooks. With regard to such a membrane the Höbers say, "Ein solche Membran wird dann die sehr bemerkenswerte Eigenschaft haben, sowohl für Anionen als auch für Kationen permeabel zu sein wird doch beide Ionen völlig zurückbehalten wofern auf den anderen Seite der Membran kein Anion oder Kation vorhanden ist das mit den erstgenannten in Austausch treten kann." Höber and Hoffmann suggest that their membrane possesses this remarkable property, but they give no experimental evidence that it is impermeable to a salt such as KCl when there are no ions on the other side which can be exchanged for the K and Cl ions to which the membrane is permeable.

Let us consider two points on such membrane as postulated, one point  $a$  on the negatively charged portion through which anions cannot pass, and one point  $b$  on that portion through which the cations cannot penetrate. The point  $a$  has a concentration\* of  $K_{1a}$  potassium ions and  $Cl_{1a}$  chloride ions on one side, and  $K_{2a}$  and  $Cl_{2a}$  on the other. For the point  $b$  the concentrations are  $K_{1b}$ ,  $Cl_{1b}$ ,  $K_{2b}$ , and  $Cl_{2b}$ . We can ascertain the conditions for equilibrium of such a system—that is for KCl not passing through the membrane. Since Cl cannot pass through  $a$ , then if K does not

$$E_{1a} - E_{2a} = RT/F \cdot \log_e [K_{2a}/K_{1a}]$$

Similarly if Cl does not pass through  $b$  and K cannot

$$E_{1b} - E_{2b} = RT/F \cdot \log_e Cl_{1b}/Cl_{2b}.$$

Since there is no traffic of K or Cl between  $a$  and  $b$  on either side of the membrane

$$E_{1a} - E_{1b} = RT/F \cdot \log_e K_{1b}/K_{1a} = RT/F \cdot \log_e Cl_{1a}/Cl_{1b}$$

$$E_{2a} - E_{2b} = RT/F \cdot \log_e K_{2b}/K_{2a} = RT/F \cdot \log_e Cl_{2a}/Cl_{2b}.$$

\* Throughout this paper we shall, for the sake of simplicity, deal with concentrations rather than activities.

As there are no other ions present, the concentration of K at any point must equal that of Cl. Hence

$$E_{1a} - E_{1b} = 0, \quad E_{2a} - E_{2b} = 0,$$

$$K_{1a} = K_{1b} = Cl_{1a} = Cl_{1b} \quad \text{and} \quad K_{2a} = K_{2b} = Cl_{2a} = Cl_{2b}.$$

Further,

$$E_{1a} - E_{2a} = E_{1b} - E_{2b},$$

and hence

$$K_{1a} = K_{2a} \quad \text{and} \quad Cl_{1a} = Cl_{2a}.$$

So the conditions for equilibrium are equality of concentration inside and out, although the nature of the membrane may be such that a long time is taken for such an equilibrium to be approached. As long as solution 1 is stronger than solution 2, then  $E_{1a}$  will be negative to  $E_{2a}$ , and  $E_{2b}$  to  $E_{1b}$ . So  $E_{1a}$  will tend to be negative to  $E_{1b}$ , and  $E_{2b}$  to  $E_{2a}$ , and we should have K ions moving to point  $a$  in the strong solution and Cl ions to point  $b$ , whilst in the weak solution the K ions would move away from point  $a$  and the Cl ions away from point  $b$ . In other words, K ions would be passing through  $a$  and Cl ions through  $b$  from the strong to the weak solution.

If the membrane as a whole was as permeable to K ions as to Cl ions, then there would be no difference of potential between the two solutions as far as points equidistant from positive and negative areas are concerned; towards the negatively charged areas the weaker solution would be positive and towards the positively charged areas negative. If the membrane, as a whole, is more permeable to K ions, whether through the negatively charged areas being of greater extent or through the mobility of the K ions being greater than that of the Cl ions, the stronger solution will be negative, but the potential difference will be less than that for a membrane completely impermeable to Cl ions. The composite membrane investigated by Höber and Hoffmann gave smaller differences of potential than did the membranes of collodion, which were practically impermeable to anions.

We can only conclude that such a membrane as the Höbers postulate will not account for the fact that potassium cannot pass out of a *Valonia* cell to sea water diluted with isotonic sugar solution.

Brooks postulates a similar type of membrane and adds: "These areas must have dimensions greater than the order of magnitude of interionic electrical forces, but need not be of visible size." The meaning of this qualification is not clear.

The accumulation of ions in the cell sap is explained by Brooks in the following manner. With the escape of carbon dioxide produced by the cell H ions escape through the areas negatively charged. The author says this necessitates the entry of other cations in amount electrically equivalent to the H ions leaving, and when equilibrium is reached the ratio of the concentration of any univalent cation inside to out must be the same as that for the H ion. Brooks continues: "In the meantime the  $\text{HCO}_3$  ion is being exchanged through the anion permeable areas for Cl, the most abundant anion present. The small accumulation of chloride in the sap (about 0.62 M. as compared with about 0.52 M. in the sea water) suggests that the penetrability of the anions present is relatively great, so that an equilibrium state is more nearly reached in their case." There is no reason to include anions other than Cl in this last statement. The author ought, by the same reasoning as he used for the cations, to have reached the conclusion, not that the inside and outside concentrations of Cl would be nearly equal at equilibrium, but that the ratio of inside to out should be the same as that for  $\text{HCO}_3$  ions. If the ratio for  $\text{HCO}_3$  is to be about unity, then, since the concentration of H ions in the sap is about a hundred times that outside, the product of the concentrations of the H and  $\text{HCO}_3$  ions, and hence the concentration of undissociated carbon dioxide, in the sap must be a hundred times that outside. Data we shall consider presently suggest the ratio for undissociated carbon dioxide is much nearer unity than a hundred.

Actually Brooks' picture is fundamentally the same as that of the Höbers; it makes no allowance for the possibility of a positive ion passing out of the cell at a negatively charged area being balanced electrically by an anion passing out of an area charged positively. Their picture would serve for the accumulation and retention of ions if it was elaborated by the addition of membranes impermeable to the electrolytes separating the cationic and anionic areas. This amounts to a colony of cells, some permeable to anions and some to cations. The former would accumulate anions and the latter cations.

No matter what the size of the areas in the mosaic membrane, the conditions at any one point inside the cell depend upon the relative resistance experienced by H and  $\text{HCO}_3$  ions in getting to the outside. If for any reason it is greater for the H ion, then that point will tend to be electrically positive to the outside, and when the ions such as K and Cl, which are neither produced nor consumed by the cell or its surroundings, reach their equilibrium distribution, then there will be a greater concentration of Cl ions at this point than outside and a lower



concentration of K ions. Conversely, where the resistance is greater for the  $\text{HCO}_3$  than for the H ions, the concentration of K ions will be greater than outside and that of the Cl ions less. Local regions close to the cationic areas may have a greater concentration of ions such as K than outside, but they will have a smaller concentration of anions such as Cl. The reverse will hold for the regions near the anionic areas of the membrane. But at all points the product of the concentrations of K and Cl ions must equal the product for the same ions outside, when equilibrium distribution is reached.

If there is any heterogeneity within the cell sap, then the products of the average concentrations of K and Cl ions inside the sap would exceed the product for the outside (Briggs and Petrie, 1928), as would the product for the average concentrations of H and  $\text{HCO}_3$  ions exceed the average concentration of carbon dioxide multiplied by its dissociation constant. Such heterogeneity within the sap could arise by wide separation of the anionic and cationic areas of the membrane, accompanied by greater ionic mobility in the membrane than in the sap. There is no evidence of such heterogeneity, and it would have to be very great to account for the high average concentrations of both anions and cations in the sap relative to outside which have been observed.

For an assimilating cell (presumably *Nitella* is when in the light) the mechanism suggested by Brooks would result in a lower, not a higher, concentration of ions in the sap than outside, since here carbon dioxide is passing into the cell, and not out.

Provided the heterogeneity is inappreciable, and carbon dioxide is passing through the membrane, the concentrations of cations such as K will tend to be greater on the side of greater concentration of carbon dioxide, if the membrane as a whole is more permeable to H ions than to  $\text{HCO}_3$  ions, and the concentration of ions such as Cl will tend to be less. The reverse will hold if the membrane is more permeable to  $\text{HCO}_3$  than to H ions.\*

\* Osterhout (1926) has put forward a scheme to account for the accumulation of KCl in *Valonia*, which is as follows:—The protoplasmic lining is assumed to be practically impermeable to K and OH ions, but permeable to KOH molecules. The KOH will tend to an equilibrium where the product of the concentrations of K and OH ions is the same inside the sap as outside. The concentration of OH ions outside is about 250 times that inside, which is kept down by the constant production of the acid  $\text{H}_2\text{CO}_3$ . Hence the concentration of K ions in the sap tends to be greater than that outside. This excess of K, and other cations, in the sap will, he says, cause an accumulation of anions such as Cl in the sap, the Cl ions going in being balanced by  $\text{HCO}_3$  ions going out. Osterhout says that penetration of KCl molecules would produce no accumulation. Actually, it would, by the same argument as he uses for KOH, lead to a smaller concentration of Cl in the

*The Suggested Mechanism—A Simple Case.*

We now proceed to consider a mechanism which would explain the accumulation of ions in the cell sap. We would emphasise at the outset that the mechanism suggested is a possible one, thermo-dynamically sound, yet not necessarily the actual mechanism. Its probability can hardly be ascertained with any precision in our present state of knowledge.

We propose to develop the case of a membrane which exhibits alternating phases of ionic permeability, thus substituting for the differences in space, of the above picture, differences in time. Membranes impregnated with proteins such as gelatin can be changed from anionic to cationic permeability by change of hydrogen ion concentration (Fujita, 1925), as can the blood corpuscle (Mond, 1927).

We will consider a simple case first. Let us suppose that carbon dioxide is being produced in a cell whose membrane is permeable only to cations, and that the cell is surrounded by a solution of KCl. As carbon dioxide is produced, some of the H ions will pass out and will be replaced by K ions, as much K passing in as H out, to balance the  $\text{HCO}_3$  left behind. If, after a certain amount of K has accumulated, the membrane was changed so that it was permeable to anions only, then the Cl would proceed to accumulate in the cell. As much Cl would pass in as  $\text{HCO}_3$  out, and  $\text{HCO}_3$  would eventually fall below H inside the cell, Cl balancing the K, and the H in excess of the  $\text{HCO}_3$ . If the cell were placed in a mixture of salts, the faster-moving ions would accumulate faster than the slower.

If the membrane is permeable to carbon dioxide, the cations would not increase indefinitely in the cationic phase. When the concentration of carbon dioxide inside has reached such a point that the rate of diffusion out equals the rate of production, then the distribution of K will tend towards an equilibrium.\* Then, since no H is passing out

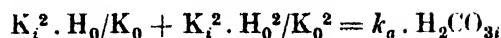
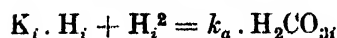
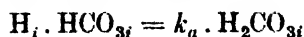
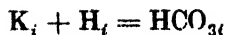
$$K_i/K_0 = H_i/H_0$$

sap than outside, since K is greater. The same condition would result if the protoplasm were permeable to H ions or HCl molecules.

The concentration of  $\text{HCO}_3$  ions is less in the sap than outside (Osterhout and Dorcas, 1925), and hence if  $\text{HCO}_3$  ions are to pass outwards against a concentration gradient, there must be a potential gradient, the outside positive to the sap. This being the case, Cl ions cannot accumulate inside the sap—that is, pass inwards against a concentration gradient and against a potential gradient.

\* It is assumed that the rate at which KCl molecules pass through the membrane is negligible.

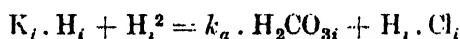
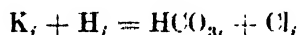
also



$$K_i = K_0 \sqrt{\frac{k_a \cdot \text{H}_2\text{CO}_{3i}}{K_0 \cdot H_0 + H_0^2}}$$

The concentration of K inside will be greater, the greater the concentration of carbon dioxide inside—that is, the greater the rate of respiration. It will increase with K outside, but the ratio  $K_i/K_0$  will fall. It will be greater the more alkaline the outside solution.

If after the second phase, the anionic phase, there is another cationic phase, then there will be Cl ions which accumulated in the second phase which cannot now get out, and so when equilibrium is reached



$$K_i^2 \left( \frac{H_0}{K_0} + \frac{H_0^2}{K_0^2} \right) = k_a \cdot \text{H}_2\text{CO}_{3i} + \text{Cl}_i \cdot K_i \cdot \frac{H_0}{K_0}$$

Hence  $K_i$  will be greater than at the end of the first phase. If these anionic and cationic phases of the membrane go on alternating, eventually a stage of equilibrium will be approached when there is no change of  $K_i$  or  $\text{Cl}_i$  on changing phase, nor will there be a change of H and  $\text{HCO}_3$  in the sap, and hence

$$K_i = K_0 \cdot H_i/H_0 \quad \text{and} \quad \text{Cl}_i = \text{Cl}_0 \cdot \text{HCO}_{3i}/\text{HCO}_{30}$$

If

$$K_0 = \text{Cl}_0$$

then

$$H_0 = \text{HCO}_{30}$$

and

$$K_i = \text{Cl}_i \quad \text{and} \quad H_i = \text{HCO}_{3i}$$

$$\frac{H_i}{H_0} = \frac{\text{HCO}_{3i}}{\text{HCO}_{30}} = \sqrt{\frac{H_i \text{CO}_{3i}}{H_2\text{CO}_{30}}} = \frac{K_i}{K_0} = \frac{\text{Cl}_i}{\text{Cl}_0}$$

Thus the ratio of the inside concentration of an ion to its outside concentration depends upon the ratio of undissociated carbon dioxide. It will be greater, the greater the rate of respiration and the smaller the outside concentration of undissociated carbon dioxide. If after having approached such a state of equilibrium the rate of respiration fell, then it would require a series of alter-

nating phases for the ratios to approach their new equilibrium values. If the ratio of  $K_0/H_0$  is of the order of 10, and after almost reaching equilibrium the ratio of  $H_2CO_3$  inside to out fall from 100 to 1.5, then in the first subsequent anionic phase the ratio  $Cl_i/Cl_0$  would fall only about 2 below 10, at which, of course, the  $K_i/K_0$  ratio would stay, since the membrane would be impermeable to cations. The fall would be less the greater the ratio of K to H in the outside solution.

The change over from anionic to cationic and the reverse need not be complete. When the membrane as a whole is more permeable to cations than to anions, the outside will be positive to the sap, with the result that K ions will accumulate in the sap. If now the membrane becomes more permeable to anions than to cations, Cl may accumulate more rapidly than the K can escape, the balance, of course, being made up by the excess of  $HCO_3$  over H which escapes. This will happen provided the relative mobility of the anions to cations in membrane becomes great enough. In this way there can be an excess of both K and Cl ions in the sap as compared with the outside solution, but the excess can not be as great as if the change over were complete. It must be noted, moreover, that this is not an equilibrium state. If one phase of permeability to both types of ion lasted long enough, then the inevitable state of equality of product of K and Cl would be reached.

If the outside solution contained a variety of ions, then in the phase predominantly anionic the faster-moving anions would accumulate more rapidly—that is, have a greater ratio inside to out provided equilibrium was not attained, and in the phase predominantly cationic the faster-moving cations would accumulate the more rapidly.

#### *The Case of Valonia.*

Osterhout and Dorcas (1925) give the total carbon dioxide of the sap of *Valonia* as  $4 \times 10^{-4}$  M, and the  $p_H$  as 5.8. This means a concentration of undissociated carbon dioxide ( $CO_2$  and  $H_2CO_3$ ) of  $3.15 \times 10^{-4}$  M. The outside values are total,  $17 \times 10^{-4}$ , and undissociated,  $0.3 \times 10^{-4}$ . The Cl in the sea water is  $560 \times 10^{-3}$  equivalents, and the K,  $12 \times 10^{-3}$ . If a *Valonia* cell were placed in sea water of such a constitution and produced carbon dioxide at such a rate as to maintain an internal concentration of  $3.15 \times 10^{-4}$  M, then after a very large number of alternating anionic and cationic phases, the former having permeability only to  $HCO_3$  and Cl, and the latter only to H and K, an equilibrium state with  $257 \times 10^{-3}$  M KCl in the sap would be approached. With the membrane permeable to Na as well as K (it can be but slightly

permeable to the former judging by its concentration in the sap) the Cl accumulation would be greater, but the K accumulation would be less. To accumulate the KCl to the extent observed (without Na) would require either a higher rate of respiration or a reduced permeability to carbon dioxide, to such an extent that the undissociated carbon dioxide was about seven times the value observed.

It should be noted that the speed at which the potassium accumulates in the cationic phase depends upon the rate at which the H ion passes out in exchange. This has to be produced by respiration, and so the accumulation depends upon the amount of respiration. The same applies to the accumulation of Cl in the anionic phase, in this case the  $\text{HCO}_3$  produced by respiration being exchanged. So the rate of production of carbon dioxide is an all important factor in determining the rate as well as the equilibrium amount of ions accumulated by the cell. Where the ions accumulate to such an extent that the osmotic pressure of the sap exceeds that of the surrounding solution, with a resulting intake of water and growth in size, rate of respiration will be an important factor determining the rate of expansion.

As the ratio of inside concentration to outside is much higher for *Nitella* than for *Valonia*, a greater ratio of carbon dioxide will be required. The effect of assimilation, which will be considered presently, would be to lower the ionic ratios. There is the possibility that the bulk of the accumulation takes place before the assimilation of the cell becomes active or after it has fallen off again. A high ratio of internal to external carbon dioxide does not necessarily mean a high rate of respiration; the rate at which it diffuses out of the cell depends upon the absolute, not the relative, difference. The concentration of carbon dioxide in the water outside will be reduced after a period of active assimilation of the cells themselves or of the surrounding cells, resulting in a temporary increase of the carbon dioxide ratio when the cell starts respiring again.

Because the concentration of K in the sap of *Valonia* is about fifty times that in the sea water, it does not follow that the ratio for the H ion concentration has been as high as this. Actually it is bigger in the mature cell. Neither does this mean that when the membrane is permeable to K ions they will pass into the sap. The rate at which ions pass into the sap depends upon the concentration gradient and upon the potential gradient.

Considering, for the sake of simplicity, univalent ions only, the rate for a cation C is

$$- \mu_c [RT dC/dx + FC dE/dx],$$

whilst that for an anion A is

$$- \mu_a [RT dA/dx - FA dE/dx],$$

where  $dE/dx$  is the potential gradient and  $\mu$  the mobility. Since the rate of movement of the anions must equal that of the cations

$$\frac{dE}{dx} = \frac{RT}{F} \cdot \frac{\sum \mu_a dA/dx - \sum \mu_c dC/dx}{\sum (\mu_a A + \mu_c C)}.$$

To obtain the value of the potential difference between sap and outside for the general case is complicated. For the simple case of mixtures of KCl and NaCl of the same total concentration, the integration is simple and the value is

$$- \frac{RT}{F} \log_e \frac{Na_i (\mu_{Na} + \mu_{Cl}) + K_i (\mu_K + \mu_{Cl})}{Na_o (\mu_{Na} + \mu_{Cl}) + K_o (\mu_K + \mu_{Cl})}.$$

A further simplification of only KCl inside and NaCl outside, a condition to which *Valonia* approaches, gives

$$- \frac{RT}{F} \cdot \log_e \frac{\mu_K + \mu_{Cl}}{\mu_{Na} + \mu_{Cl}}.$$

This is the special case worked out by Henderson (1907). The problem is much simpler if the membrane is permeable to but one type of ion, anion or cation. In the former case

$$E_i - E_o = RT/F \cdot \log_e [\sum \mu_a A_i / \sum \mu_a A_o],$$

in the latter

$$E_i - E_o = - RT/F \cdot \log_e [\sum \mu_c C_i / \sum \mu_c C_o].$$

The rate at which potassium ions move into the sap is

$$- \mu_K [RT dK/dx + FK dE/dx].$$

Assuming that the membrane of *Valonia* is permeable only to those ions in the sap—namely, H, K, Na, Cl and  $HCO_3$ —we are faced with the question as to whether the quantity  $-dE/dx$  is greater or less than  $RT/FK \cdot dK/dx$ . If it is greater, then K will pass in and, if it is less, out.

$$- \frac{dE}{dx} = \frac{RT}{F} \cdot \frac{\mu_H \frac{dH}{dx} + \mu_{Na} \frac{dNa}{dx} + \mu_K \frac{dK}{dx} - \mu_{Cl} \frac{dCl}{dx} - \mu_{HCO_3} \frac{dHCO_3}{dx}}{\mu_H H + \mu_{Na} Na + \mu_K K + \mu_{Cl} Cl + \mu_{HCO_3} HCO_3}.$$

In the absence of knowledge of the relative mobilities of the various ions we cannot give a definite answer. All we can say is that since  $dCl/dx$  is positive

(more Cl in the sap than outside) and  $dNa/dx$  is negative, these will both tend to make  $-dE/dx$  less, and hence tend to make K pass out of the sap, whilst the H and  $HCO_3$  gradients will have the opposite effects. In a purely cationic phase

$$-\frac{dE}{dx} = \frac{RT}{F} \cdot \frac{\mu_H \frac{dH}{dx} + \mu_{Na} \frac{dNa}{dx} + \mu_K \frac{dK}{dx}}{\mu_H H + \mu_{Na} Na + \mu_K K}.$$

The small value of the ratio of Na inside to out (less than one-fifth), compared with that for K (nearly 50), suggests that the mobility of Na in the membrane is very small compared with that of K. If it is small enough to render the effect of the Na gradient negligible, then, since the mobility of H is probably greater than that of K and  $dH/dx$  is greater than  $dK/dx$ , the gradient  $-\frac{dE}{dx}$  or  $\frac{RT}{F} \cdot \frac{\mu_H dH/dx + \mu_K dK/dx}{\mu_H H + \mu_K K}$  will be greater than  $\frac{RT}{F} \cdot \frac{dK/dx}{K}$  and K will pass in. It all depends upon whether the mobility of Na is small enough.

Since the rate at which Cl will pass in is

$$-\mu_{Cl} \left[ RT \frac{dCl}{dx} - FCl \frac{dE}{dx} \right]$$

it will pass in or out according as to whether  $dE/dx$  is greater or less than  $\frac{RT}{F} \cdot \frac{dCl/dx}{Cl}$ . In the general case, if K is passing in then  $dE/dx$  must be negative, and Cl will be passing out, since  $dCl/dx$  is positive. In a purely anionic phase

$$\frac{dE}{dx} = \frac{RT}{F} \cdot \frac{\mu_{Cl} \frac{dCl}{dx} + \mu_{HCO_3} \frac{dHCO_3}{dx}}{\mu_{Cl} Cl + \mu_{HCO_3} HCO_3},$$

and since  $dHCO_3/dx$  is negative  $dE/dx$  must be less than  $RT/FCl \cdot dCl/dx$ , and hence Cl will pass out in exchange for  $HCO_3$ .

The above holds for sea water with a  $p_H$  about 8. As the excess base is of the order of  $10^{-3}$  equivalents per litre, the  $HCO_3$  would not be reduced to a concentration smaller than that in the cell sap of *Valonia* (taking the figures of Osterhout and Dorcas) until the  $p_H$  of the sea water was increased to about 11.

If the membrane were permeable to H and K ions only when in the cationic phase, then the cell sap would tend to an equilibrium where

$$H_i = \frac{Cl_i + \sqrt{Cl_i^2 + 4k_a \cdot H_o CO_{2i} (1 + K_o/H_o)}}{2(1 + K_o/H_o)},$$

neglecting anions other than Cl. With  $\text{H}_2\text{CO}_3$  in the sap at  $3 \times 10^{-4}$  and  $K_0/\text{H}_0$  at about  $12 \times 10^5$  and Cl, 0.6 the value for  $\text{H}_i$  is  $5 \times 10^{-7}$ . So the  $p_{\text{H}}$  of the sap instead of staying at 6, its usual value, it should drift to 6.3 in the cationic phase.

If the membrane in the anionic phase were permeable to Cl and  $\text{HCO}_3$  only, the cell sap would tend to an equilibrium where

$$\text{H}_i^2 + \text{H}_i (\text{K}_i + \text{Na}_i) - k_a \cdot \text{H}_2\text{CO}_{3i} (1 + \text{H}_0\text{Cl}_0/k_a \cdot \text{H}_2\text{CO}_{30}) = 0$$

or since  $\text{H}_i$  is so small

$$\text{H}_i = \frac{\text{H}_2\text{CO}_{3i}}{\text{H}_2\text{CO}_{30}} \cdot \frac{\text{H}_0 \cdot \text{Cl}_0}{(\text{K}_i + \text{Na}_i)}$$

With  $\text{H}_2\text{CO}_3$  in the sap about 10 times that outside and  $\text{H}_0 \cdot \text{Cl}_0$  at  $5.6 \times 10^{-9}$  and  $\text{K}_i$  plus  $\text{Na}_i$  at 0.6, the value of  $\text{H}_i$  tends to  $9 \times 10^{-8}$ ; more alkaline than in the cationic phase.

With a continuation of alternating anionic and cationic phases (with impermeability to Na) an equilibrium with about 0.09 M NaCl and about 0.21 M KCl with a  $p_{\text{H}}$  of 6.95 would be approached, instead of 0.09 M NaCl, 0.52 M KCl and  $p_{\text{H}}$  6.

The production of some acid other than  $\text{H}_2\text{CO}_3$  by the cell would, of course, interfere with these calculations and would tend to keep the sap at a higher acidity, at the same time depressing the ionisation of the carbon dioxide.

As we shall see later, Osterhout and his co-workers record only positive values for the P.D. between the sap and the sea water, thus suggesting that the membrane is in an anionic phase. It would be interesting to know whether the P.D. is independent of such changes as the *Valonia* is likely to experience under natural conditions; whether it is the same in light and dark, at different concentrations of H ions in the surrounding medium, in young cells and in older.

Little would be gained by a detailed consideration of the case of *Nitella* along the above lines.

Various writers have drawn attention to the fact that on the whole the ionic ratios, inside to out, are in the order of the ionic mobilities in free solution, anions and cations being considered separately. For example, the ratio for K exceeds that for Na, and that for the latter is greater than that for the bivalent cations Ca and Mg, whilst that for Cl exceeds that for  $\text{SO}_4$  ions. As we pointed out earlier, the order of ionic mobilities in membranes which have been investigated is the same as that for free solution, as far as the above ions are concerned.



As Collander (1930) points out, the agreement of ratios and mobilities is not exact. In *Chara*, which he investigated, the ratio for K is 63, that for Na 2.4, but that for Ca is bigger, 2.9, and that for Mg is as big as that for Na. It should be noted that when the ions reach an equilibrium distribution, it is not the ratios which should be equal but the  $n$ th root of the ratio, where  $n$  is the valency of the ion. The rate at which the cation passes into the sap is

$$- \mu_c \left[ RT \frac{dC}{dx} + nFC \frac{dE}{dx} \right].$$

So that, if the potential gradient favours the movement in, increased valency may make up, more or less, for decreased mobility. The square root of the Ca and Mg ratios is less in both cases than the Na ratio, so the results are not really abnormal, as Collander suggests. This writer also points out that the ratio for  $\text{NO}_3$  and  $\text{PO}_4$  is higher than for the fast-moving Cl ion. In *Chara*, even the cube root of the  $\text{PO}_4$  ratio exceeds the Cl ratio. Neither is this state of affairs abnormal. When the anions are accumulating, a fast-moving anion may approach its equilibrium value more rapidly than a slow-moving ion, but when the conditions are changed so that the ratios are decreasing, then the ratio for the fast-moving ion will tend to fall more rapidly than that for the slow moving. If, before the change, the ratio for both fast moving and for slow was near the equilibrium value, then after the change the slow-moving ion would tend to have the greater ratio. This applies to cations as well as to anions. A change of rate of respiration would provide the cause for the lowering of the ratio. The ratio for all the ions of one type, univalent anions for example, need not be inverted. Two fast-moving ions may have approached nearly to their equilibrium values before the change took place, whilst the ratio for a very slow-moving ion might still be very small, so that after the change the less fast of the two fastest may have the biggest ratio, the fastest the next, and it may not yet have fallen as low as the ratio of the slowest, which may still be rising. We must conclude, then, that perfect correlation of ionic mobility and ratio of internal to external concentration is not to be expected, since change of rate of output of carbon dioxide is most likely.

#### *Effect of Photosynthesis on Ionic Accumulation.*

According to Brooks, *Valonia* lives in places where the light is so feeble that the rate of photosynthesis is likely to be negligibly small. Osterhout and Dorcas record that the total concentration of carbon dioxide in the cell sap changed from  $4 \times 10^{-4}$  M in the dark to  $1.8 \times 10^{-4}$  M after 5 hours' illumi-

nation (no intensity stated), whilst that outside changed from  $17 \times 10^{-4}$  to  $14 \times 10^{-4}$  M. Generally, they maintain that the  $p_H$  of the sap is remarkably constant at about 6. If this is so, in light and dark, then in the dark the undissociated carbon dioxide ( $H_2CO_3$  plus  $CO_2$ ) in the sap is about  $3 \times 10^{-4}$  M, while that outside is about  $0.5 \times 10^{-4}$ , and in the light that for the sap is  $1.35 \times 10^{-4}$ , and that in the sea water  $0.4 \times 10^{-4}$  M. This would mean that carbon dioxide is passing out of the cell in the light as well as in the dark.

There is no suggestion, as far as we are aware, that cells of *Nitella* and *Chara* do not take in carbon dioxide when illuminated sufficiently.

Passage of carbon dioxide into a cell with alternating anionic and cationic phases of the membrane, such as we have postulated, would tend to a lower concentration of both positive and negative ions in the sap as compared with the outside solution. The presence of ions in the sap which could not get out would cause disturbances, just as they would in the case where carbon dioxide is being produced, and not consumed. Apart from such complications our original equation

$$\frac{K_i}{K_o} = \frac{Cl_i}{Cl_o} = \sqrt{\frac{H_2CO_{3i}}{H_2CO_{3o}}}$$

would hold when equilibrium was reached. So electrolytes would tend to accumulate in a cell, when respiration was greater than assimilation, and pass out when the reverse held.

If, however, the anionic phase of the membrane coincided with the intake of carbon dioxide by the cell, and the cationic with the output (or *vice versa*), it would be possible to have both the anion and the cation ratios greater than unity when equilibrium was reached. Another way would be to have impermeability to all ions in the light when carbon dioxide was passing into the cell. The equations for the former case are readily formulated, but not so easily solved. For the following numerical example: External solution H,  $5 \times 10^{-7}$ ;  $HCO_3$ ,  $5 \times 10^{-7}$ ; K,  $10^{-5}$ ; Cl,  $10^{-5}$ ; and the anionic phase coinciding with a consumption of carbon dioxide of such magnitude that the internal concentration is 2/25ths of the outside, and the cationic phase coinciding with a production of carbon dioxide producing an internal concentration 27 times that outside; an equilibrium state is approached with K and Cl in greater concentration inside than out. It does not seem profitable to pursue this further until we know more about the changes of permeability with age of cell, and change of external conditions.

*Experiments involving Change of the Ionic Composition of the External Solution.*

We have now to consider the results of experiments in which the effect of change in the ionic composition of the external solution has been investigated.

Brooks (1929) investigated the effect of placing *Valonia* in sea water modified by the addition of isotonic KCl or NaCl, by comparing the sap with that from cells in normal sea water. Unfortunately some of his experiments were rendered rather ineffective by the accidental use of 0.476 M KCl instead of 0.597 M; an error for which the author tries to make allowance. In one extreme the K content of the sea water was reduced to half of the control value, and the Na put up by 11 per cent., whilst in the other the K was increased eight-fold, and the Na reduced to 85 per cent. of the control. With the addition of KCl it is stated that the Cl content of the sea water was unchanged. In that case an addition of an equal quantity of 0.597 M NaCl ought to increase the Cl content of the sea water by 12 per cent., and not 7 per cent. as Brooks states.

The effect of these modifications on the sap is very small, and it is doubtful if the differences between the changed and the control cells have any great significance. Nevertheless, Brooks claims that in all cases the K increased in the sap and Na decreased relative to the control. Now this does not mean that the concentration of the K in the cells actually increased or that the concentration of the Na decreased, as the author states. It is difficult to conceive of the mechanism whereby, at the same time, K passes inwards against a concentration gradient and Na passes outwards also against a concentration gradient. Any potential gradient enabling K to pass in against the concentration gradient would cause Na to pass in, as the potential and concentration gradients would work together for the Na ions. The conclusion really is, that modification of the sea water enables K to accumulate more rapidly and Na less rapidly than in the control, or that the rate of loss of K is decreased more than that of Na. Brooks also states that Cl passes into the sap. Again, it is really that more passes out, or less passes into the controls compared with the experimental.

Let us consider only those experiments where the Na and Cl of the sea water were increased and the K decreased—that is, where no change was made in the total concentration. If the membrane is not affected by the change, we should expect, in the cationic phase, a greater increase of Na and a smaller increase of K relative to the control; perhaps the K might decrease. The potential of the sap will be made more negative to the outside by the substitution of the

slower-moving Na for K outside. This will minimise the concentration effect. The net uptake of cations would be reduced by the substitution of a slower-moving cation for a faster. In the anionic phase less Cl will escape, or more will go in if the outside  $\text{HCO}_3$  is low enough. This will reduce the decrease in uptake of total cation in the cationic phase, assuming the phases alternate. The loss of Cl will be increased, or the uptake decreased, by the smaller uptake of total cations consequent upon the substitution of the slow Na for K. It is impossible to say what the net result will be; it depends upon the duration of phases, among other things. We might have either an increase of total cations and Cl relative to the control, or a decrease. But, in any case, the Na ought to increase more, or decrease less, than the control.

If the Cl increases, we should have to postulate an effect on the membrane to explain the increase of K and decrease of Na relative to the control. The change in the sea water may reduce the mobility of Na relative to that of K in the membrane. This would favour uptake of K relative to Na and might actually give the desired result, if the change in relative mobilities is great enough. Whether the phases of the membrane alternate or not, an effect on the membrane of this type seems essential to explain the results which Brooks claims his experiments give.

We have not mentioned possibilities such as the effect of the change of sea water on the rate of respiration, and hence upon the rate of change of the ions in the sap, or the effect of the change on the permeability to Cl ions.

Assuming that the membrane has both anionic and cationic phases, the negligible change of K, observed by Höber and Höber, when the cells were placed in sea water mixed with isotonic sugar, might well be due to the short duration of the experiment and the few tests made. As we have pointed out already, the change of K for H must be slow. The exchange of Cl for Br in the sap when the latter is substituted for the former outside is free from complication. The rapid exchange is to be expected, since the mobilities of Cl and Br are very similar in free solution, and there was plenty of Br to replace the Cl.

The experiments of Hoagland and Davis (1922, 1923) with *Nitella* are more complicated, since the cells were usually placed in phosphate solutions of  $p_{\text{H}} 5$  (very different from their normal environment) along with salts.

They record that cells placed in 0.005 M KCl plus 0.005 M  $\text{CaCl}_2$  had, after 28 days, 3,500 parts per million of K in the sap and 4,720 of Cl. If the cells, when placed in the solution, possessed sap like that given for cells from pond water, then both positive and negative ions have accumulated against

concentration gradients, and hence there must have been both anionic and cationic phases of the membrane, according to our suggested picture. Since the only ions outside are K, Ca, and Cl, some of the K may have gone in at the expense of Na in the sap and some of the Cl at the expense of the  $\text{SO}_4$ . But there is hardly enough of the other anions in the sap to account for the increase of the Cl by exchange, and it seems that  $\text{HCO}_3$  may have passed out. In this case, the amount of carbon dioxide and hydrogen ion in the external solution are factors of importance, since pond water, at  $p_{\text{H}}$  7 or 8, in equilibrium with atmospheric carbon dioxide, would most likely have a greater concentration of  $\text{HCO}_3$  ions than the sap.

Other experiments show that Cl ions are removed from solution more rapidly when the chloride is in the form of KCl or RbCl than when in the form of  $\text{CaCl}_2$  or  $\text{MgCl}_2$ . This may mean greater mobility of Cl ions in the presence of K or Rb than in the presence of Ca or Mg. A more likely explanation is that the greater accumulation of cation, on account of greater mobility, in the cationic phase would result in the greater accumulation of any anion in the ensuing anionic phase. It is not clear whether the cells were placed in pure salt solutions or in the buffer solution, plus the salt. In the former case, it would be more difficult to get the outside concentration of  $\text{HCO}_3$  low enough for it to be less than the inside, so that Cl could pass into the sap when the membrane was in the anionic phase.

It was found that increase of the acidity of the outside solution markedly increased the rate of penetration of anions, such as  $\text{NO}_3$ , into the sap. The passage of  $\text{NO}_3$  into the sap during the anionic phase will be dependent upon other anions passing out. The increase of the acidity might increase the permeability of the membrane to the ions, and thus facilitate the interchange. It seems much more likely that the bulk of the uptake of the  $\text{NO}_3$  is at the expense of  $\text{HCO}_3$  produced by the cell. With the  $p_{\text{H}}$  of the sap at 5 and that of the outside at 7, the  $\text{HCO}_3$  of the sap may well be less than that outside, if the latter is in equilibrium with the atmosphere: to be greater, the concentration of carbon dioxide in the sap must be more than a hundred times that in the solution outside. After a period of photosynthetic intake of carbon dioxide, the external carbon dioxide may be very small, and the  $\text{HCO}_3$ , for a time, smaller than that inside during the following period of respiratory output of carbon dioxide. This would be more likely if the cells were kept in closed beakers of unstirred water, as was probably the case, no mention being made of stirring. In any case, increase of the acidity of the outside would result in a depression of the ionisation of the carbon dioxide, and a consequent

lowering of the  $\text{HCO}_3$  outside, with an increase in the chance of the inside concentration of that ion exceeding the outside. In this way increased acidity would facilitate the accumulation of anions.

Addition of 0.005 M KCl or KBr to a solution containing 0.005 M  $\text{KNO}_3$  resulted in a reduction in the uptake of  $\text{NO}_3$  ions, whereas  $\text{K}_2\text{SO}_4$  had little retarding effect. This would be expected, if the mobility of Cl and Br in the membrane was greater than that of  $\text{NO}_3$  and that of  $\text{SO}_4$  (allowing for its valency) were less. Such is the state of affairs in free solution and in artificial membranes. The faster-moving anion would pass into the sap in exchange for the  $\text{HCO}_3$  coming out at the expense of the accumulation of the slower-moving anion.

Finally, there are the results showing greater accumulation of anions in the light than in the dark—results which led the authors to suggest that energy supplied by radiation was used in some unknown way in doing work in accumulating the ions. Accumulation of an anion without the corresponding loss of another anion, apart from  $\text{HCO}_3$ , in a cell continuously illuminated and consuming carbon dioxide, would be difficult to explain. Fortunately, perhaps, there is no evidence of such happening. Faster interchange of ions in light than dark might be interpreted as increased permeability of the membrane in the light. Light would work in other ways. Increased assimilation would mean decreased  $\text{HCO}_3$  outside, and hence a bigger gradient of this ion outwards in the ensuing period of respiration. If this period was accompanied by an anionic phase of the membrane, then uptake of anions from the outside would be facilitated by previous illumination. Further, the photosynthesis would provide carbohydrates, and these would tend to increase the rate of respiration and thus further increase the gradient of  $\text{HCO}_3$  outwards.

As we have pointed out before, permeability to anions while the cell was consuming carbon dioxide would mean a passage of  $\text{HCO}_3$  ions into the cell, and hence there would be tendency for other anions to pass out.

There are many other ways in which light might have an effect. For example, increased permeability in a cationic phase would lead to increased accumulation of cations, which would have its accelerating effect on anion accumulation in the anionic phase, quite apart from any effect of light during this phase.

In many of the experiments, what was measured was removal from external solution, which may well be different from passage into sap.

In concluding this section we may say that all the results fit in quite well with a picture of anionic and cationic phases of the membrane around a cell,

which is capable of producing and consuming carbon dioxide on suitable occasions, provided the relative mobilities of the ions in the membrane are what we should expect from the mobilities in free solution and in artificial membranes. The results with *Valonia* could be explained by assuming that the membrane of the mature cell is permeable to anions only, the cations having accumulated at an earlier stage of development.

*The Potential Difference between the Sap and the Outside Solution.*

Experiments by Osterhout and his co-workers (1927) have shown that for *Valonia* and *Nitella* the sap inside is electrically positive to sap supplied to the outside. These results lead the authors to the conclusion that the protoplasmic membrane is three-layered, the layer to the outside being different from that facing the sap. The nature of the difference is not indicated.

There can be no difference in potential between the sap inside and out if of identical composition, whatever the nature of the membrane (provided it is doing no work), when equilibrium is reached. A small difference in concentration of carbon dioxide would cause a negligible difference of potential in the presence of salts about 0.6 M.

When the *Valonia* cell is in sea water the inner side of the membrane is in contact with a sap roughly 0.5 M KCl plus 0.1 M NaCl, and the outside with roughly 0.56 M NaCl, plus 0.12 M KCl. If the outside solution is changed to sap, then K ions will tend to pass into the membrane from the outside faster than before and Na less rapidly, or the latter may actually come out. Similarly, the Cl will pass in less rapidly or come out. The greater mobility of the K ion relative to the Na ion, and the greater concentration change of the K as compared with Cl, will probably leave the K as the deciding ion, and in this case the outside sap will be left negative to the membrane, and hence to the inside sap, until equilibrium is reached. So there is no need to postulate a difference in the membrane surfaces to explain a temporary difference of potential between sap inside and sap outside; the difference can rest in the difference in the two solutions outside and inside the membrane previously. Of course, the two sides of the membrane are different in their ion content.

The P.D. between the sap of *Valonia* and the surrounding sea water is stated to be 4 or 5 millivolts. If the membrane is permeable to anions only, then the sap should be positive to an extent decided by

$$\frac{RT}{F} \log \frac{\mu_{\text{HCO}_3} \text{HCO}_{3i} + \mu_{\text{Cl}} \text{Cl}_i}{\mu_{\text{HCO}_3} \text{HCO}_{3o} + \mu_{\text{Cl}} \text{Cl}_o}.$$

The concentration of  $\text{HCO}_3$  ions is so small compared with that of the Cl ions that, unless the mobility of the former is very great compared with that of the latter, the  $\text{HCO}_3$  can be left out of account. The ratio of the Cl ion concentrations would account for a P.D. of less than 2 millivolts. If the membrane were permeable to cations only, then the P.D. would be

$$-\frac{RT}{F} \log_e \frac{\mu_H H_i + \mu_K K_i + \mu_{Na} Na_i}{\mu_H H_o + \mu_K K_o + \mu_{Na} Na_o}.$$

For the sap to be positive, the mobility of the Na ions would have to be three times as big in the membrane as that of the K ions. Mobilities in free solution and in artificial membranes, and also the ratios of Na and K ions (sap to sea water), all point to K mobility in the membrane being the greater. Hence a positive P.D. of sap to sea water suggests that under the experimental conditions the membrane is permeable to anions only.

It is difficult to understand a P.D. as high as 4 or 5 millivolts, and all the figures recorded are with one exception bigger than 4. There is one possibility: in this particular table the units are not stated, and earlier in the paper the electrometer scale was said to be 1.5 to 2 metres per volt, so perhaps the units are millimetres and not millivolts!

If only anions pass right through the membrane, and, as we suggested above, cations pass into the outer part when the sea water is replaced by sap, then, either the inner part of the membrane is impermeable to cations, and so different from the outer part, or the outer part of the membrane is rendered permeable to cations when sap takes the place of sea water. In the above, outer part of membrane may perhaps be cell wall.

In view of our previous suggestions, it would be interesting to know if the P.D. between sap and sea water was the same in light and dark and whether, when equilibrium was established, it was affected by changing the relative amounts of Na and K in the sea water.

In the experiments with *Valonia* contact was made with the inner cell sap directly, but those with *Nitella* were different. Cell sap was applied at two points, A and C. The P.D. between A and C was small, but, when cell sap with chloroform was applied at C, then A became negative. As evidence is produced that the P.D. across killed protoplasm is negligible, the inner sap can be said to be positive to the outer. This difference can be explained in just the same way as was that for *Valonia*.

The sap is also positive to 0.1, 0.05 and 0.01 M KCl and negative to 0.001 M and more so to 0.001 M NaCl. All these results can be explained on the above



lines; in the former cases more cations passing into the outer membrane surface than out, or less anions, and the reverse in the last two. The difference between the last two might be due to the greater passage out of K in the one case, owing to its greater mobility if to nothing else, than of Na in the other. A knowledge of the composition of the water to which the *Nitella* cells had previously been exposed is necessary before we can say whether the above is a sufficient explanation.

There are no data, as far as we are aware, for the P.D. between sap and solutions in which the cells naturally grow. It is such data which are of particular interest, in that they throw light on the question as to whether the membrane is more permeable to anions or cations. The experiments involving change of outside solution, in as far as they are of short duration, only give us information about penetration into the outer layers of the membrane.

#### *Summary.*

The suggestions that a membrane composed of a mosaic of areas, some permeable to anions, and some to cations, will (a) not let either ions of a salt pass through if there are no ions on the other side to exchange with them, and (b) explain the accumulation of both anions and cations in cells, are discussed. Both neglect the fact that ions passing out through one type of area can be balanced electrically by ions of the opposite charge passing out through the other type of area.

A possible schema, based on alternating phases, one of greater permeability to anions and the other of greater permeability to cations, is suggested to explain the accumulation of ions.

Existing data on the distribution of ions between the sap and outside solution, and on the potential difference between the two, are discussed.

#### REFERENCES.

- Briggs and Petrie (1928). 'Biochem. J.,' vol. 22, p. 1071.  
Brooks (1929). 'Protoplasma,' vol. 8, p. 389.  
Collander (1930). 'Acta Botanica Fennica,' vol. 6.  
Fujita (1925). 'Biochem. Z.,' vol. 162, p. 245.  
Henderson (1907). 'Z. Physik. Chem.,' vol. 59, p. 118.  
Hoagland and Davis (1922). 'J. Gen. Physiol.,' vol. 5, p. 629.  
Hoagland and Davis (1923). 'J. Gen. Physiol.,' vol. 6, p. 47.  
Höber and Höber (1928). 'Pflüger's Arch.,' vol. 219, p. 260.  
Höber and Hoffmann (1928). 'Pflüger's Arch.,' vol. 220, p. 558.  
Michaelis (1927). 'Colloid Symposium Monograph,' vol. 5.

- Mond (1927). 'Pflüger's Arch.,' vol. 217, p. 618.  
 Mond and Hoffmann (1928). 'Pflüger's Arch.,' vol. 220, p. 194.  
 Osterhout, Damon and Jacques (1927). 'J. Gen. Physiol.,' vol. 11, p. 193.  
 Osterhout (1926). 'Proc. Soc. Expt. Biol. and Med.,' vol. 24, p. 234.  
 Osterhout and Dorcas (1925). 'J. Gen. Physiol.,' vol. 9, p. 255.  
 Osterhout and Harris (1927). 'J. Gen. Physiol.,' vol. 11, p. 391.  
 Osterhout and Harris (1927). 'J. Gen. Physiol.,' vol. 11, p. 673.

541 . 182 : 535 . 435.

*The Scattering of Light in Protein Solutions. I.—Gelatin Solutions and Gels.*

By K. KRISHNAMURTI, D.Sc., University College, London.

(Communicated by F. G. Donnan, F.R.S.—Received May 26, 1930.)

(Abstract.)

(1) The effect of temperature on the intensity and depolarisation of the light scattered by gelatin sols and gels has been investigated; and a view regarding the nature of gelation has been put forward. Gelatin sols have been considered to be polydisperse systems, in which part of the gelatin is present in the molecularly dispersed condition, and the rest as polymolecular micells. Cooling the sol below 25° produces supersaturation, as a result of which fresh particles are formed, and these become larger due to the condensation of the molecularly dispersed gelatin on them.

(2) An ultracentrifugal examination of gelatin sols at  $p_H$  4.8 shows clearly that a considerable aggregation of gelatin molecules takes place at this  $p_H$ . This is also supported by an ultramicroscopic examination.

(3) During the gel-sol transformation the gel-micells are gradually dispersed into the original molecular condition.

(4) The molecular state of gelatin in the sols is discussed, and reasons are put forward to justify the use of the term "molecules" for the protein particles.

(5) The variation of the intensity of the scattered light with the concentration of gelatin sols and gels has been examined. At about 40° the Tyndall number increases with concentration up to 4 per cent. gelatin content, above which it

remains constant. But, when these sols are cooled to about  $10^{\circ}$  the dilute sols are much more turbid than the concentrated ones. An explanation of this observation has been offered.

(6) A careful examination of the variation in the depolarisation ( $\theta$ ) of the scattered light when gelatin sols of different concentrations are cooled to  $10^{\circ}$ , reveals certain interesting facts.  $\theta$  decreases at first and then increases; and this effect is more pronounced in the case of dilute sols. The significance of this phenomenon in revealing the changes in the size and shape of the micells has been pointed out.

(7) Judging from the parallelism between the Tyndall intensity and the sedimentation constant in gelatin sols at different  $p_H$  values it is clear that the turbidity near the isoelectric point is caused by the aggregation of gelatin molecules. The author is inclined to the view that the effectiveness of acid or base in preventing the precipitation of gelatin is due to the capacity of acid or alkali to combine with gelatin, thereby increasing its solubility, so that lowering of the temperature does not cause a supersaturation of the solution and the precipitation of gelatin.

The author desires to express his best thanks to Prof. F. G. Donnan, C.B.E., F.R.S., for his very kind interest in this work.

[*The full paper is printed in 'Proceedings,' Series A, vol. 129, pp. 490-508.*]

*Prothrombase—Its Preparation and Properties.*

By JOHN MELLANBY, F.R.S.

(From the Physiological Laboratory, St. Thomas's Hospital, London.)

(Received August 29, 1930.)

An attempt to reconcile the various hypotheses which have been advanced to account for the phenomena observed in the coagulation of blood necessitated the isolation from blood of those substances which are primarily involved in the coagulation process. In this paper the preparation of prothrombase from oxalated mammalian blood is described and the conditions under which it is converted into thrombase—the enzyme which converts fibrinogen into fibrin—are considered.

In 1909 I described a method by which a solution of prothrombase may be obtained from birds' blood. Noncoagulable plasma is obtained from a fasting anaesthetised cockerel in the way described by Delezenne (1897). This plasma is diluted with 10 volumes of distilled water and brought to the isoelectric point for globulin by the cautious addition of acetic acid (1 per cent.). The precipitated globulin is obtained as a compact mass by spinning the diluted plasma in a high-speed centrifuge. It is suspended in a volume of water equal to that of the original plasma and dissolved by the addition of NaCl to the extent of 0·7 per cent. This solution contains prothrombase associated with fibrinogen. The addition to it of a little tissue extract (thrombokinase), and  $\text{CaCl}_2$  to the extent of 0·05 per cent. causes, within a few minutes, the formation of a solid coagulum of fibrin. The residual fluid, obtained after the removal of the fibrin, contains a large quantity of thrombase. If, however, a small quantity of thrombase be added to the original fibrinogen-prothrombase solution coagulation takes place within a few seconds and the residual fluid, after removal of the fibrin, contains a large quantity of prothrombase. The properties of avian prothrombase contained in such a solution were described. No attempt was made to isolate the prothrombase owing to the difficulty of obtaining stable noncoagulable birds' blood in large quantities.

(A) *The Preparation of Prothrombase from Mammalian Blood.*

The main principles for the preparation of prothrombase from birds' blood have now been applied with success to oxalated mammalian blood. An extension of the method has enabled preparations of prothrombase of considerable potential activity to be isolated. The method finally adopted for the isolation of prothrombase is first described. In a subsequent section the difficulties which may arise in the course of the method are described.

(a) Mammalian blood (ox) is received into bottles (2 litre) containing 20 c.c. of 20 per cent. neutral potassium oxalate, and the contents are thoroughly mixed. The final concentration of oxalate is 0.2 per cent. This large excess of oxalate usually ensures that the plasma, obtained from the blood by high-speed centrifugalisation, remains fluid for at least 3 days. Sometimes a partial clot forms in the oxalated plasma in the course of 48 hours owing to the delay in mixing the oxalate with the shed blood, but this partial coagulation diminishes to a small degree only the prothrombase content of the remaining plasma.

(b) The plasma is diluted with 10 volumes of distilled water, and brought to the isoelectric point for prothrombase ( $p_H$  5.3) by the addition of acetic acid. About 35 c.c. of acetic acid (1 per cent.) requires to be added to each litre of diluted fluid containing 100 c.c. of plasma. This figure may vary considerably in certain cases. The maximum precipitation of globulin from the diluted plasma does not correspond with the maximum precipitation of prothrombase. Until this fact was recognised considerable difficulties were encountered in the uniform preparation of prothrombase from different varieties of plasma. The precipitate in the dilute acidified plasma rapidly flocculates and the greater portion of it settles to the bottom of the vessel in 2 hours. The supernatant fluid is then poured off and the dilute suspension of globulin is spun in a high-speed centrifuge. By this means the precipitate is obtained as a compact mass and may be resuspended, in a fine state of division, in a volume of distilled water equal to half that of the original plasma.

(c) The analysis of the precipitate from the diluted plasma shows that it contains prothrombase, fibrinogen, serum globulin, pigment and cholesterol. The removal of prothrombase only from this complex presents considerable difficulties. The following method gives the best results:—

Lime water [ $\text{Ca}(\text{OH})_2$  0.168 per cent.], i.e., water saturated with calcium oxide at room temperature ( $16^\circ \text{C}$ .), is diluted ten-fold with distilled water and brought to a reaction of  $p_H$  7 by cautious treatment with  $\text{CO}_2$ . The concentration of Ca in this neutral fluid is about 0.0092 per cent. This dilute

calcium bicarbonate solution is now added to an equal volume of the prothrombase-fibrinogen suspension. The two fluids are mixed by gentle shaking and allowed to stand for about 10 minutes. At the end of this time, during which there is no apparent solution of the precipitate, the suspension is rapidly filtered through a series of coarse filter papers. It is advisable to filter a large volume of suspension through a number of small filter papers to effect rapid filtration. Apparently the prothrombase is split rapidly from the globulin complex and passes into solution. If, however, the calcium bicarbonate solution be left in contact with the precipitate for a long period of time, first the serum globulin and then the fibrinogen tend to pass into solution. The essential difficulty of the method depends on the determination of the point at which the prothrombase passes into solution in the dilute calcium bicarbonate and leaves the other substances (fibrinogen, serum globulin, pigment, etc.) still undissolved.

(d) The solution of prothrombase prepared in this way should be water clear and of a volume approximately equal to that of the original plasma. The addition of kinase and  $\text{CaCl}_2$  to a small portion of it should produce a thrombase solution of which 0.1 c.c. coagulates 1 c.c. of oxalated plasma in 10 seconds. The prothrombase is precipitated from the rest of the solution by the addition to it of HA 1 per cent. until the reaction is approximately  $p_H$  5.3. At this degree of acidity the whole of the prothrombase is precipitated and may be obtained as a compact mass by spinning in a centrifuge.

(e) The precipitate may be quickly dried by treatment with acetone. The final product is a white amorphous powder which retains its potential activity for an indefinite period of time when kept in a dry state. Its potency, when estimated in terms of the capacity of the activated substance to clot oxalated plasma, varies somewhat with different preparations. When converted into thrombase 0.02 mgrm. of the average preparation coagulates 1 c.c. of oxalate plasma in 10 seconds.

The difficulty in obtaining preparations with a uniform potency may depend upon the association of prothrombase with serum globulin. All attempts to fractionate to a greater degree the prothrombase prepared by the above method have failed. It is possible that prothrombase obtained from different animals varies somewhat in its composition and that this variation determines the lack of uniformity in the activity of the prothrombase preparations.

The average yield from 100 c.c. of plasma is approximately 40 mgrm., a quantity which when converted into thrombase coagulates approximately 2 litres of oxalate plasma in 10 seconds. The large quantity of prothrombase

in plasma ensures rapid and efficient coagulation when injured tissue (kinase) enters the blood.

(B) *Notes on the Various Stages in the Preparation of Prothrombase.*

(a) *Dilution.*—The oxalated plasma should be diluted with water to bring the salt concentration below 0·1 per cent. In some cases large quantities of oxalate are added to the blood by inadvertance. In these cases a 15-fold dilution of the plasma is advisable to ensure the precipitation of the prothrombase.

(b) *Reaction.*—It is essential that the reaction of the diluted plasma should be brought to about  $p_H$  5·3 by the addition of acetic acid. The importance of the reaction of the diluted plasma is exemplified in the following experimental results.

Dilute plasma (10 c.c. plasma added to 90 c.c.  $H_2O$ ) was placed in a series of tubes and brought to varying reactions by the addition of acetic acid. The precipitates were obtained by centrifugalisation, suspended in a volume of water equal to that of the original plasma, dissolved in NaCl 0·7 per cent., and brought to a neutral reaction by the addition of  $Na_2CO_3$  (0·5 per cent.). The solutions were coagulated by the addition of kinase and  $CaCl_2$  and after 15 minutes the thrombase content of the expressed fluids was determined. In the table the first column gives the reaction of the dilute acidified plasma, and the second column gives the prothrombase content of the precipitate in terms of thrombase.

Reaction of dilute plasma ( $p_H$ ).	Coagulation time for 0·1 c.c. activated prothrombase added to 1 c.c. oxalate plasma
	seconds
6·5	60
6·02	30
5·76	22
5·40	18
5·28	8
5·25	12

\* I am indebted to Mr. N. F. MacLagan of the Courtauld Institute of Biochemistry, Middlesex Hospital, W.1, for these figures.

The figures show that there is an optimum reaction for the precipitation of prothrombase from diluted oxalate plasma. In the above experiment there was a definite resolution of the precipitated globulin at a reaction of  $p_H$  5·25. It is essential to recognise therefore that failure to precipitate prothrombase from the diluted plasma may be due to adding either too little or too much acid.

(c) *The Resolution of Prothrombase from the Globulin Precipitate.*—The precipitate obtained from the dilute acidified plasma is a complex mixture. The greater portion of it consists of prothrombase, fibrinogen and serum globulin, but the pigments of serum, cholesterol and thrombokinase are always present in variable quantities. The separation of prothrombase from this complex precipitate has presented considerable difficulties.

(i) The addition of calcium bicarbonate to a suspension of the globulin precipitate in water results in the splitting of the prothrombase from the complex. But only a minimal quantity of calcium must be used to prevent the subsequent conversion of the liberated prothrombase into thrombase and the further change of the fibrinogen into fibrin. The quantity of calcium in the solution used is approximately 0.009 per cent., a concentration which separates prothrombase only from the complex and which is too small to activate the liberated prothrombase in the period of time necessary for filtration and subsequent precipitation by acid. The protocols of two experiments are appended to illustrate the general method and to show that the optimal quantity of calcium bicarbonate solution is approximately half that of the original volume of plasma.

400 c.c. of oxalated ox plasma were added to 4000 c.c. of distilled water: 140 c.c. HA 1 per cent. were added to the diluted plasma to bring the reaction to approximately  $p_H$  5.3. The mixture was allowed to stand for 2 hours during which time the precipitate flocculated and settled to the bottom of the cylinder. The supernatant fluid was poured off and the precipitate obtained as a compact mass by spinning in a high-speed centrifuge. This precipitate was ground up in a mortar with a little distilled water until it was in a fine state of division and then the suspension was made up to 200 c.c., i.e., half the volume of the original plasma. The solution of calcium bicarbonate was now made by adding 20 c.c. of lime water to 180 c.c. of distilled water and cautiously bubbling  $CO_2$  through the fluid until the reaction was  $p_H$  7. The following dilutions of the suspension and the bicarbonate solution were now made :—

Suspension.	Calcium bicarbonate.	$H_2O$ .
c.c.	c.c.	c.c.
40	10	50
40	15	45
40	20	40
40	30	30
40	40	20



After 10 minutes the suspensions were rapidly filtered through coarse folded filter papers : 60 c.c. of each filtrate were treated with HA (0.1 N) until the reaction was approximately  $p_H$  5.3. The precipitated prothrombase was obtained by spinning, washed several times with acetone, and dried in a vacuum dessicator.

The yields in each case and the activities of the products when converted into thrombase are given in the following table :—

Yield.	Coagulation time on adding 0.012 mgrm. of activated precipitate to 1 c.c. oxalate plasma.
mgrm.	seconds
15	20
20	15
24	14
30	15
35	13

The figures show that the best yield of prothrombase was obtained when the calcium bicarbonate solution was equal in volume to the fibrinogen suspension. They also show that the prothrombase split off by smaller quantities of calcium bicarbonate is rather less active than that obtained with larger quantities.

An experiment similar to the above was made on another sample of plasma in which the amounts of calcium bicarbonate used were much greater than in the above experiment, *i.e.*, the ratios of bicarbonate to globulin suspension were (a) 1 : 1, (b) 2 : 1, and (c) 3 : 1. The yields of prothrombase and the activities of the products when converted into thrombase were :—

Yield.	Coagulation time on adding 0.012 mgrm. of activated prothrombase to 1 c.c. oxalate plasma.
mgrm.	seconds
(a) 40	15
(b) 58	25
(c) 67	28

It is evident from these figures that the best prothrombase preparation was obtained by extracting the globulin complex with an equal volume of the calcium bicarbonate solution. The larger yields with greater volumes of bicarbonate were correspondingly poorer in prothrombase content.

During the course of this work the capacities of many compounds to split the prothrombase from the globulin complex have been determined. It may

facilitate future work if the results obtained in these experiments are briefly mentioned. The reaction of the globulin complex suspended in water is definitely acidic ( $p_H$  5.3). Therefore attempts were made to dissolve the prothrombase contained in it by treating a suspension in water with various alkaline solutions.

(ii) NaOH.—Quantities of NaOH varying from 1:100,000 to 1:10,000 were added to suspensions of the globulin complex in water. On a few occasions a strong solution of prothrombase containing only a small quantity of fibrinogen was obtained. Usually however, the fibrinogen tended to pass into solution with the prothrombase. The results were too uncertain to allow the method to be adopted as a standard procedure.

(iii)  $Na_2CO_3$ .—More consistent results were obtained by extracting the globulin complex with  $Na_2CO_3$  (0.1 per cent.). On many occasions solutions of prothrombase, contaminated only by traces of fibrinogen or serum globulin, were obtained. But in this case also considerable variations were encountered which could not be controlled.

(iv)  $BaCO_3$ .—The addition of solid barium carbonate to a suspension in water of the globulin complex (about 10 mgrm.  $BaCO_3$  to each 100 c.c. of suspension) results in the prothrombase passing into solution. In many cases the prothrombase is comparatively pure, being accompanied only by the pigment of the complex. This solution of prothrombase is comparatively stable and remains unchanged even after standing for several days at room temperature.

(v)  $CaCO_3$ .—The addition of solid calcium carbonate to a suspension in water of the globulin complex (about 10 mgrm.  $CaCO_3$  to each 100 c.c. of suspension) results in the prothrombase passing into solution. But in a comparatively short period of time the greater part of the prothrombase is converted into thrombase, and this, in turn, acts on the fibrinogen, converting it into fibrin. It was impossible to overcome these difficulties until the method of using a very dilute solution of calcium bicarbonate was adopted.

(vi) *Neutral Salts*.—The globulin complex is insoluble in water but soluble in dilute solutions of neutral salts. Many attempts were made to fractionate the complex by partial solution in neutral salts such as NaCl,  $Na_2SO_4$ ,  $MgCl_2$ ,  $MgSO_4$  and  $CaCl_2$ . No success was attained by any of these methods. In this connection it is of interest to observe that neutral solutions of  $CaCl_2$  in concentrations varying from 0.01 N to 0.1 N are unable to split prothrombase from the globulin complex. Two factors are apparently required for this purpose: (1) the neutralisation of the acid contained in the globulin complex to bring the reaction to approximately  $p_H$  6.8; and (2) the action of the Ca ion

in dissociating the prothrombase from the globulin complex. Both these conditions are satisfied by the dilute solution of calcium bicarbonate described in the method finally adopted for the preparation of prothrombase.

(d) *Precipitation of Prothrombase from Solution by Acid.*—Prothrombase is completely precipitated from solution at a reaction of  $p_H$  5·3. Greater degrees of acidity redissolve the prothrombase and precipitation is incomplete at less acidic reactions. Fractional precipitation by increasing quantities of acid does not lead to the concentration of prothrombase in any one fraction. Further, fractional resolution of the precipitated prothrombase by sodium carbonate does not concentrate the prothrombase in any one fraction. In fact, if prothrombase is associated with a foreign protein in the product prepared by this method fractional treatment with acid or alkali does not lead to the concentration of prothrombase in any one fraction.

(e) *Drying.*—Prothrombase precipitated from solution by acid is a very stable substance. It is unaffected by alcohol, ether, or acetone. As a routine method the prothrombase may be conveniently dried by washing several times with acetone. Usually some of the pigment of the plasma is associated with the prothrombase in its final precipitation, and this is readily removed by acetone, leaving a white amorphous powder. The preparation is finally dried in a vacuum dessicator. The product is stable and may be left for an indefinite period of time in the dry state without any diminution of its potential activity.

### (C) *The Activity of Various Preparations of Prothrombase.*

Prothrombase preparations obtained from the oxalated plasma of different animals vary in their potential activities. The following experimental results were obtained, with four products obtained at different seasons of the year. In each case 5 mgrm. were dissolved in 50 c.c. of water containing a trace of  $Na_2CO_3$ . These solutions were activated by the addition of kinase and  $CaCl_2$  and dilutions corresponding to 0·01 mgrm. and 0·001 mgrm. of the original prothrombase preparations were added to 1 c.c. of oxalate plasma and the times of coagulation observed.

#### Coagulation time of oxalate plasma.

	0·01 mgrm.	0·001 mgrm.
(1)	15 seconds	2 minutes
(2)	15 seconds	1·75 minutes
(3)	15 seconds	2 minutes
(4)	25 seconds	3 minutes.

The first three preparations had approximately the same activity, but preparation (4), compared with (1), (2) and (3) was relatively impure. The figures, however, emphasise the great activity of the preparations when tested on oxalate plasma. Attempts to purify preparation (4) by fractional solution and precipitation were unsuccessful. It has been observed in later experiments that inactivation of thrombase by heat does not alter its physical properties as regards solubility and precipitation limits. Possibly therefore the variable activity of the preparations may depend to some extent upon previous activation and inactivation, rather than on the presence of associated impurities.

#### (D) *The Properties of Prothrombase.*

Prothrombase is a white amorphous powder of an acidic nature. When converted into thrombase it coagulates *in vitro* approximately 100,000 times its weight of oxalate plasma within 20 seconds.

(a) *Chemical Composition.*—A dilute solution of prothrombase (0.05 per cent.) gives well-marked protein reactions (buiuret, xanthoproteic, millon, glyoxylic, ninhydrin). The powder (5 mgrm.) gives a definite sulphide reaction with nitroprusside after heating with sodium. On the other hand after the wet combustion of 25 mgrm. with  $\text{HNO}_3$  and  $\text{H}_2\text{SO}_4$  only a very faint phosphate reaction is obtained. Comparative tests with casein indicated that the phosphate content is less than 0.02 per cent.

(b) *Solubility.*—Prothrombase is insoluble in such organic solvents as acetone, alcohol and ether. It is practically insoluble in water. It is, however, readily dissolved when sodium carbonate is added to the water in which it is suspended until the reaction rises to about  $p_{\text{H}}$  7.

(c) *Destruction by Heat.*—Prothrombase suspended in water and heated to  $100^\circ \text{C}$ . is destroyed. This treatment makes it insoluble in dilute sodium carbonate, and the addition of kinase and  $\text{CaCl}_2$  does not lead to the formation of thrombase. On the other hand a solution of prothrombase may be heated to  $100^\circ \text{C}$ . without destruction although the facility with which this heated prothrombase is converted into thrombase is very much diminished. This fact is illustrated by the following experimental results :—

1 c.c. of a solution of prothrombase adjusted to a reaction of  $p_{\text{H}}$  8 was put into a series of tubes. Each of these tubes was maintained at a definite temperature for 5 minutes. After this time the tubes were cooled to  $40^\circ \text{C}$ . and activated by the addition of optimal quantities of kinase (0.1 c.c.) and  $\text{CaCl}_2$  (1 : 2000).

The rates of activation were determined by adding 0.1 c.c. of the activating mixtures to 1 c.c. of oxalate plasma after varying times.

Temperature to which the prothrombase was heated.	Coagulation times (in seconds) on adding 0.1 c.c. to 1 c.c. of oxalated plasma after varying times (in minutes).										
	1 min.	2 min.	5 min.	7 min.	9 min.	10 min.	15 min.	20 min.	25 min.	30 min.	45 min.
° C.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
40	10	—	—	—	—	—	—	—	—	—	—
50	—	10	—	—	—	—	—	—	—	—	—
60	—	—	58	16	10	—	—	—	—	—	—
70	—	—	—	—	50	30	10	—	—	—	—
80	—	—	—	—	50	30	10	—	—	—	—
90	—	—	—	—	—	—	—	36	20	20	15
100	—	—	—	—	—	—	—	60	30	20	20

Two facts are evident from these results: (1) heating a solution of prothrombase to 100° C. for 5 minutes diminishes to a small degree only its capacity to form thrombase when subsequently treated with kinase and calcium chloride; and (2) the extraordinary resistance to activation produced by the previous heating to a high temperature. In the unheated prothrombase solution maximal activation was produced in 1 minute; in the solution which had been heated to 100° C. maximal activation was produced only after 45 minutes.

(d) *Dialysis*.—A solution of prothrombase was placed in a collodion dialyser surrounded by a large volume of distilled water which was often renewed. After definite intervals of time 1 c.c. of this solution was taken from the collodion tube and activated by the addition of kinase and calcium chloride. The prothrombase content after 5 days was the same as at the beginning of the experiment. The results show: (1) the inability of prothrombase to pass through a collodion membrane and (2) the stability of prothrombase in dilute solution at room temperature. It is of interest to observe that the prothrombase showed no tendency to activate spontaneously.

(e) *Proteolytic Enzymes*.—It is impossible to test the action of pepsin or trypsin on prothrombase since concentrations of acid or alkali in which these enzymes react (0.2 per cent. HCl and 0.5 per cent. Na<sub>2</sub>CO<sub>3</sub>) rapidly destroy prothrombase at 38° C.

A consideration of these above properties of prothrombase indicates that prothrombase is an acid meta protein, or is associated with an acid meta protein upon which the preservation of its properties depends.

(E) *The Activation of Prothrombase by Thrombokinase and Calcium Salts.*

The addition of tissue extract alone (thrombokinase) to a solution of prothrombase always causes the generation of a corresponding quantity of thrombase in the course of a few hours. This fact holds good even when the prothrombase has been prepared from solutions containing no calcium salts, *i.e.*, oxalate plasma and the replacement of calcium bicarbonate by sodium bicarbonate in the second stage of the process. It appears, therefore, that kinase alone is able to activate prothrombase. The presence of minute quantities of calcium salts has, however, a marked effect on the velocity of activation, so much so that no definite statement on the place of calcium salts in the reaction may be made until thrombokinase has been isolated in a pure condition.

(a) *Kinase varying, Calcium constant (1 : 2000).*—Solutions were made containing a definite quantity of prothrombase and calcium chloride, but varying quantities of kinase. After definite intervals of time the thrombase contents of these mixtures were determined by observing the time taken by 0.1 c.c. to coagulate 1 c.c. of oxalate plasma.

Pro.	H <sub>2</sub> O.	CaCl <sub>2</sub> (N/5).	K.	Coagulation times (seconds) on adding 0.1 c.c. to 1 c.c. plasma after varying times (minutes).				
				1 min.	2 min.	3 min.	5 min.	10 min.
c.c.	c.c.	c.c.	c.c.	sec.	sec.	sec.	sec.	sec.
1	0.8	0.1	0.1	58	25	17	10	—
1	0.85	0.1	0.05	—	30	14	12	10
1	0.88	0.1	0.02	—	56	20	15	12
1	0.89	0.1	0.01	—	—	80	20	12
1	0.895	0.1	0.005	—	—	70	50	18

These figures show that the velocity of activation is slow at first but proceeds with increasing acceleration, a fact which has previously been described for avian prothrombase and also for the generation of trypsin from trypsinogen by enterokinase. The results also indicate that a minimal quantity of kinase is required to produce a maximal activation of prothrombase, *i.e.*, that a quantitative relation may exist between prothrombase and kinase to produce a maximum quantity of thrombase. But I propose to deal more fully with this point when considering the properties of thrombase.

(b) *Calcium varying, Kinase constant.*—Experiments were carried out

(similar to that just described) in which quantities of prothrombase and kinase were kept constant whilst the quantities of calcium chloride were varied. The following figures illustrate the results obtained.

Pro.	K.	H <sub>2</sub> O.	CaCl <sub>2</sub> (N/50).	Coagulation time (seconds) on adding 0.1 c.c. to 1 c.c. plasma after varying times (in minutes).									
				1 min.	2 min.	3 min.	5 min.	10 min.	15 min.	20 min.	25 min.	30 min.	35 min.
(i)	c.c.	c.c.	c.c.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
	1	0.1	0	50	16	12	—	—	—	—	—	—	—
	1	0.1	0.4	83	16	12	—	—	—	—	—	—	—
(iv)	1	0.1	0.8	—	—	48	12	—	—	—	—	—	—
	1	0.1	0.85	—	—	—	—	—	—	120	90	35	15

Experiments (i) and (iv) illustrate the great effect produced by diminishing the CaCl<sub>2</sub> from a concentration of 1 : 2000 to 1 : 40,000. In the former case complete activation was produced in 3 minutes ; in the latter case in approximately 35 minutes. The addition of kinase alone to the above prothrombase solution produced no thrombase after the lapse of 2 hours. The reaction of the mixture was about  $p_H$  7. But when a fresh solution was made up and the reaction was adjusted  $p_H$  8 the addition of kinase only resulted in complete activation within 45 minutes, i.e., in approximately the same time as that required in experiment (iv) in which the activating mixture contained CaCl<sub>2</sub> (N/2000).

(c) *The Effect of Reaction on the Rate of Activation of Prothrombase.*—A solution containing 7.5 c.c. of prothrombase, 0.5 c.c. of kinase and 2 c.c. of CaCl<sub>2</sub> (N/500) was divided into five equal parts and adjusted to different reactions. The rates of activation, determined on oxalate plasma, are shown in the following figures :—

Coagulation times (seconds) of 0.1 c.c. added to 1 c.c. plasma after varying times.

Reaction.

	min.	20 min.	25 min.	30 min.	1 hr.
$p_H$	sec.	sec.	sec.	sec.	sec.
6	—	—	—	—	—
7	—	33	14	10	—
8	—	50	15	12	10
9	—	60	20	13	10

It is evident that the generation of thrombase from prothrombase by kinase and calcium salts does not proceed in acid solution, and that the optimal reaction is approximately neutral. It is interesting to observe, however, that a change in reaction from  $p_H$  7 to  $p_H$  9 delays to a small degree only activation of prothrombase. Possibly the fact that the precipitation of prothrombase from solution starts at  $p_H$  6.7 may account for the absence of activation by kinase and calcium salts in an acidic medium.

(d) *The Influence of Temperature on the Rate of Activation of Prothrombase.*—A solution containing prothrombase (9 c.c.), kinase (0.5 c.c.) and  $\text{CaCl}_2$  N/5 (0.5 c.c.) was divided into five equal portions and maintained at different temperatures. The relative rates of activation are shown in the following results :—

Coagulation time (seconds) on adding 0.1 c.c. to 1 c.c. of plasma after varying times (minutes).						
Temperature.	2 min.	3 min.	4 min.	5 min.	10 min.	20 min.
° C.	sec.	sec.	sec.	sec.	sec.	sec.
15	—	—	65	50	16	13
25	40	20	15	12		
35	20	15	12	—		
45	20	15	12	—		
(x) 55	25	no coagulation.				

The results indicate (1) the rapid rate at which prothrombase is converted into thrombase at a temperature so low as 15° C. ; (2) the comparative absence of a temperature coefficient between 25° C. and 45° C. ; and (3) the rapid destruction of thrombase at 55° C. In the solution (x) maintained at 55° C. the thrombase produced after 2 minutes' activation was completely destroyed at the end of the third minute. This is in marked contrast to the stability of prothrombase at 100° C. as evidenced by the results described in a previous section (p. 279).

(F) *A Comparison of the Effects of Calcium, Strontium, Barium and Magnesium on the Activation of Prothrombase by Thrombokinas.*

The addition of calcium chloride to oxalate plasma in excess of the quantity of oxalate contained in it results in coagulation within about 3 minutes (38° C.). Similar results are obtained when strontium chloride is substituted for calcium chloride. But the addition of barium chloride or magnesium chloride to



oxalated plasma never results in coagulation, i.e., barium and magnesium ions are unable to replace calcium ions in the activation of prothrombase contained in plasma. It was of interest therefore to compare the relative effects of these ions on the activation of prothrombase, isolated from the other constituents of plasma. The following results give the rates of activation of solutions of prothrombase containing kinase and  $\text{CaCl}_2$ ,  $\text{SrCl}_2$ ,  $\text{BaCl}_2$  and  $\text{MgCl}_2$  to the extent of N/50. The reaction was adjusted to  $p_H$  8 in every case.

Pro.	K.	$\text{H}_2\text{O}$ .	Salt.	Coagulation times on adding 0.1 c.c. to 1 c.c. plasma after varying times (minutes).		
				2 min.	10 min.	30 min.
c.c.	c.c.	c.c.	c.c.	sec.	sec.	sec.
1	0.1	0.8	0.1 $\text{CaCl}_2$	10	—	—
1	0.1	0.8	0.1 $\text{SrCl}_2$	10	—	—
1	0.1	0.8	0.1 $\text{BaCl}_2$	—	72	26
1	0.1	0.8	0.1 $\text{MgCl}_2$	—	50	15
1	0.1	0.9	0	—	—	90

The results indicate that all these salts accelerate the activation of prothrombase by thrombokinase. But with barium and magnesium the degree of acceleration is very small and these ions take about twenty times as long as calcium or strontium to produce maximal activation. This relatively slow rate of activation by barium and magnesium probably allows other processes to enter into the reaction in plasma and these prevent the slowly formed thrombase from coagulating the fibrinogen. Hence the absence of coagulation when salts of barium are added to oxalate plasma in excess of the oxalate contained in it.

#### (G) Summary.

1. Prothrombase may be prepared from oxalated mammalian plasma by (a) precipitation at a reaction of  $p_H$  5.3, of the globulin complex from the plasma diluted with 10 volumes of water; (b) extraction of the prothrombase from this complex by a dilute solution of calcium bicarbonate; and (c) precipitation of the prothrombase from the calcium bicarbonate solution by the addition of acid to a reaction of  $p_H$  5.3.

2. The yield of prothrombase is about 40 mgrm. from 100 c.c. of plasma.

3. 1 mgrm. of the average prothrombase preparation when converted into thrombase coagulates 100 c.c. of oxalate plasma in 20 seconds. This fact in

conjunction with (2) above explains the rapid coagulation of plasma when kinase (injured tissue) enters the blood stream.

4. Prothrombase is a white amorphous powder which gives the characteristic reactions of acid meta protein. It is insoluble in water but freely soluble in dilute alkali. It is not dialysable through a collodian membrane, nor is it destroyed when an aqueous solution of it is heated to 100° C. for 5 minutes. This latter procedure, however, diminishes the rate at which it may be converted into thrombase.

5. Prothrombase may be activated by the addition to it of thrombokinase, but the rate of generation of thrombase is greatly accelerated by the presence of small quantities of calcium. The rate of activation is slow at the beginning of the reaction, but proceeds with a rapidly increasing velocity. The velocity of activation is modified by the reaction of the solution and the temperature.

6. Sr, Ba or Mg may replace Ca in the activation of prothrombase by thrombokinase. Sr is as effective as Ca in the activation process, but the efficiency of Ba or Mg is very small. With these two ions (Ba and Mg) activation proceeds at a rate only slightly greater than that produced by thrombokinase alone.

#### REFERENCES.

- Delezenne (1897). 'Arch. de Physiol.,' p. 333.  
Mellanby (1909). 'J. Physiol.,' vol. 33, p. 28.

*The Porphyrin of Component c of Cytochrome and its  
Relationship to other Porphyrins.*

By R. HILL (Beit Memorial Research Fellow), and D. KEILIN, F.R.S.

(From Biochemical Laboratory and Molteno Institute, Cambridge.)

(Received August 21, 1930.)

*I. Introduction.*

Of the hæmatin compounds present in the cell, component *c* of cytochrome alone can be readily obtained in solution by extraction with water of either dry or acetone yeast (Keilin, 1925). Recently a method for extracting and concentrating this pigment from yeast has been described, and it was found that the component *c* thus obtained has the same properties as it has in intact living cells or in the extracts of dry or acetone yeast cells (Keilin, 1930, pp. 421-423). It was shown previously that cytochrome *c* is a hæmochromogen which differs from the usual hæmochromogen compounds in two important properties, namely, in not being autoxidisable and in not forming a carbon monoxide compound. It was also shown that, without changing its absorption spectrum, the component *c* can be easily modified in such a way as to show the properties of an ordinary hæmochromogen (Keilin, 1925, 1926). As the various hæmochromogens obtained from cytochrome *c* have the positions of the absorption bands very different from those of protohæmochromogens it was clear that the iron-porphyrin portion of its molecule is completely different from the ordinary protohæmatin. A series of facts discussed in previous papers suggested, however, that cytochrome originates from the ordinary protohæmatin which is also present in all cells of aerobic organisms.

The object of the experiments described in this paper is the study of the porphyrin portion of cytochrome *c* and its relationship to other porphyrins, especially to protoporphyrin.

It is important to note that the cytochrome *c* preparations obtained, as was previously described (1930)\*, were completely free from ordinary hæmatin

\* For extraction of cytochrome *c* on a larger scale, 2 kg. of plasmolysed yeast are added to 5 litres of boiling water. After 5 minutes' warming, when 90° C. is reached, 5 kg. of broken ice are added. When the temperature drops to 25° C. any unmelted ice is removed to accelerate sedimentation. The preparation is completed as previously described, the ingredients added being proportional to the amount of yeast used.

compounds especially from protohæmatin, as the spectrum of protohæmochromogen could never be detected when pyridine and a reducer were added to the solutions of *c*. Moreover, the component *c* used for the preparation of porphyrin was always that which showed no direct reaction with oxygen or carbon monoxide. The concentration of pigment in our preparation was estimated by comparing its colour and absorption spectrum with those of known standard solutions of pyridine mesohæmochromogen. The results thus obtained were confirmed by the iron estimation (total iron minus the free inorganic iron). By these methods the pigment preparation, when concentrated and dried, in addition to proteins, was found to contain 0.6 per cent. of hæmatin (assuming the molecular weight of the latter to be 640).

Two different methods have been used for the preparation of porphyrins. The first method consisted in treating cytochrome with acetic acid saturated with HBr. The porphyrin thus obtained was indistinguishable from ordinary hæmatoporphyrin obtained in the same way from protohæmatin. It was found also that this porphyrin, like ordinary hæmatoporphyrin, can be converted into protoporphyrin.

The second method consisted in treating the component *c* with aqueous hydrochloric acid and  $\text{SO}_2$ . The porphyrin obtained by this method, which we shall call porphyrin *c*, was found to be different from all known porphyrins; it seems to correspond more closely to that which would be considered as the natural porphyrin of the component *c* of cytochrome.

## *II. Preparation of Hæmatoporphyrin by the Action of Hydrobromic and Acetic Acid on the Component c of Cytochrome.*

The brown precipitate of cytochrome preparation (Keilin, 1930, p. 422) was dried either in a desiccator or by rapidly washing in acetone. The dry mass was pulverised and 0.5 g. of powder was introduced into a small 20 c.c. stoppered bottle to which was added 10 c.c. of acetic acid saturated with dry hydrobromic acid. The mixture was thoroughly shaken and after standing overnight 2 drops of water were added until all the proteins were dissolved giving a clear purplish brown solution. This fluid was then diluted with 5 volumes of water and, after the addition of sodium acetate, the porphyrin was extracted with ether and acetic acid. The porphyrin from the ether layer was extracted with 3N. HCl and after the addition of sodium acetate, passed again into ether, and from ether it was transferred to 3N. hydrochloric acid. The porphyrin obtained by this method was similar both in position

of absorption bands and colour of its solutions in acid and in ether with pure hæmatoporphyrin obtained from protohæmatin.

The absorption bands of the porphyrin were perfectly sharp and, compared with ordinary hæmatoporphyrin occupied the following positions :—

	Bands.				
	I	II	III	IV	V
1. <i>In ether and acetic acid</i> —					
Hæmatoporphyrin prepared from protohæmatin .....	6250	5975	5681	5295	4960
Hæmatoporphyrin prepared from cytochrome <i>c</i> .....	6250	5975	5689	5295	4955
2. <i>In N . HCl</i> —					
Hæmatoporphyrin prepared from protohæmatin .....	5934		5500		
Hæmatoporphyrin prepared from cytochrome <i>c</i> .....	5935		5500		
3. <i>In aqueous pyridine</i> *				$\alpha$	$\beta$
Copper compound of hæmatoporphyrin prepared from protohæmatin .....				5630	5260
Copper compound of hæmatoporphyrin prepared from cytochrome <i>c</i> .....				5625	5260
4. <i>Pyridine hæmatohæmochromogen</i> —					
Pyridine hæmatohæmochromogen prepared from protohæmatin .....				5468	5175
Pyridine hæmatohæmochromogen prepared from cytochrome <i>c</i> .....				5465	5165

The hæmatin prepared from hæmatoporphyrin (*c*) combines with the native globin and gives the oxyhæmoglobin compound the absorption spectrum of which is similar to that obtained by Hill and Holden (1926) from the ordinary hæmatoporphyrin derived from hæmatin.

5. <i>Hæmatohæmoglobin</i> —	$\alpha$	$\beta$
Hæmatohæmoglobin prepared from protohæmatin.....	5682	5325
Hæmatohæmoglobin prepared from cytochrome <i>c</i> .....	5680	5320

\* Cu hæmatoporphyrins were extracted with pyridine from the aqueous phase by addition of excess of ammonium sulphate.

### III. Conversion of the *Hæmatoporphyrin* obtained from the Component *c* of *Cytochrome* into *Protoporphyrin*.

Three methods have been used for the conversion of hæmatoporphyrin of cytochrome *c* into protoporphyrin. As control experiments porphyrin obtained from ordinary hæmatin was treated simultaneously and in exactly the same way.

1. The first method—that of Willstätter and M. Fisher (1913)—which was extensively used by H. Fisher (1925) consisted in heating the free porphyrins *in vacuo*. About 1 mg. of the hæmatoporphyrin *c* dissolved in 2 c.c. of ether and acetic acid was placed in a small tube (A) and the ether was evaporated off. A small amount of ordinary hæmatoporphyrin was treated in the same way (B). The small tubes containing the two porphyrins were placed in two larger tubes drawn out at one end and connected to a Geryk vacuum pump. The two tubes, while still under the action of the pump, were heated in calcium chloride bath at 140° to 145° C. for 15 minutes. After cooling, the contents of the tubes were mixed with a drop of water and dissolved in ether and acetic acid. The ether extract, after being washed with water, in both tubes showed a mixed spectrum of proto- and hæmatoporphyrin. On the addition of M/5 HCl to the ether all the unmodified hæmatoporphyrin was found in the water phase, leaving in each case most of the protoporphyrin in the ether layer.

The positions of the absorption bands of the ether layer in both tubes compared with those of unmodified hæmatoporphyrin and of protoporphyrin were as follows :—

	Bands.				
<i>In ether and acetic acid—</i>	I	II	III	IV	V
Hæmatoporphyrin from cytochrome <i>c</i> . .	6250	5975	5689	5295	4955
Protoporphyrin from ordinary hæmatoporphyrin (tube B) . . . . .	6323	6055	5745	5366	5022
Protoporphyrin from hæmatoporphyrin <i>c</i> (tube A) . . . . .	6323	6055	5750	5366	5020
Ordinary protoporphyrin . . . . .	6325	6045	5757	5366	5022

2. The second method consisted in heating the hæmatohæmatins *in vacuo*. The porphyrins were combined with iron in the usual way and, after the addition of 3N. HCl, the hæmatins were extracted with ether, transferred into two small tubes and treated in exactly the same way as in the former experiment with the porphyrin. After heating *in vacuo* the hæmatins were dissolved

in dilute sodium hydroxide. One portion of each was treated with pyridine and  $\text{Na}_2\text{S}_2\text{O}_4$ , the other was combined with native globin. In each case the absorption bands occupied the same positions and were intermediate between bands of compounds prepared from proto- and hæmatoporphyrins.

3. The third method was based on the observation of Bois (1927) that hæmatoporphyrin is converted into protohæmatin on long heating with acetic acid and iron wire. Iron was combined with hæmatoporphyrin *c* by the usual method using acetic acid and a ferrous salt, and when the pigment was transformed into acid hæmatin the fluid was boiled in a small tube (A) and immediately sealed. The tube was heated for 2 hours to  $140^\circ \text{C}$ . The contents of the tube was then mixed with an equal volume of water and an excess of pyridine (2 volumes). The hæmochromogen bands were measured after reduction with sodium hydrosulphite. As a control experiment, ordinary hæmatohæmatin was treated in the same way (tube B).

The positions of absorption bands of the pyridine hæmochromogens of the initial and final products compared with protohæmochromogen in presence of excess of pyridine were as follows :—

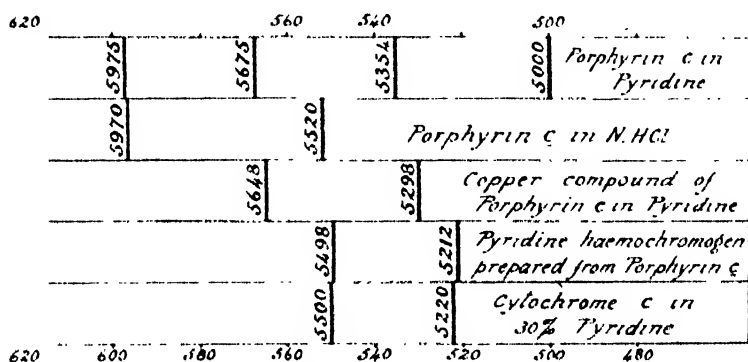
Pyridine hæmochromogens prepared from :

	$\alpha$	$\beta$
A. Hæmatohæmatin (obtained from cytochrome <i>c</i> ) . . . . .	5465	5165
Product of A after heating . . . . .	5529	5232
B. Hæmatohæmatin (obtained from protohæmatin) . . . .	5468	5175
Product of B after heating . . . . .	5530	5235
C. Protohæmatin . . . . .	5550	5251

#### IV. *Preparation of the Unmodified Porphyrin from Component c of Cytochrome.*

The iron can be removed from component *c* of cytochrome by treatment with aqueous hydrochloric acid and a reducing agent. 3 c.c. of a solution of component *c* containing 0.2 mg. of hæmatin was added to 3 c.c. of concentrated HCl. This gave a brown fluid in which the proteins were just in solution and which showed the absorption spectrum of acid hæmatin. A current of  $\text{SO}_2$  was passed through and the fluid was kept in a Thunberg tube from which the air was evacuated. After standing overnight the fluid turned purple in colour and showed a typical acid porphyrin spectrum. On addition of an excess of acetic acid and ether the porphyrin was precipitated in the form of dark purple liquid droplets. On dissolving in water and adding sodium acetate no porphyrin could be extracted with ether and acetic acid,

all the pigment remaining dissolved in the acetic acid-aqueous layer and showing a typical spectrum of alkaline porphyrin. No pigment could be extracted by chloroform. The porphyrin, however, could be extracted with pyridine by adding pyridine to the hydrochloric acid solution saturated with ammonium sulphate. All the pigment passed then into the pyridine forming the upper layer separated from the colourless aqueous phase. The porphyrin of cytochrome *c* obtained by this less drastic treatment was found to give a pyridine hæmochromogen corresponding in the positions of its absorption bands to that obtained directly from component *c* treated with pyridine (see figure).



The figure shows the positions of the absorption bands of porphyrin *c* in pyridine and in 10 per cent. HCl, of its copper compound in pyridine and its pyridine hæmochromogen compared with the component *c* of cytochrome dissolved in pyridine.

### V. Summary and Conclusions.

1. Two different porphyrins can be prepared from the unmodified cytochrome *c* obtained from bakers' yeast cells.

2. Under the action of hydrobromic and acetic acid, cytochrome *c* yields a hæmatoporphyrin which is indistinguishable from hæmatoporphyrin prepared in the same way from the ordinary hæmatin. Both show the same solubilities in ether and acetic acids, and the same absorption spectra in ether and in hydrochloric acid. Both when reunited with Fe combine with various nitrogen compounds or with native globin and give respectively hæmochromogens or hæmoglobins with similar absorption spectra.

3. Both hæmatoporphyrins (prepared from cytochrome *c* and from ordinary hæmatin) can be easily converted into protoporphyrin. This conversion can be accomplished by three different methods: (a) by heating the hæmatoporphyrin *in vacuo*; (b) by heating the corresponding hæmatohæmatin *in vacuo*; or (c) by heating the hæmatohæmatin in acetic acid in a sealed tube.



4. The fact that hæmatoporphyrin obtained from *c* can be converted into protoporphyrin gives an additional support to the supposition previously proposed (Keilin, 1926) as to the origin of cytochrome, namely, that it is derived from the protohæmatin present in cells. This supposition was based so far upon the following observations : (a) that in addition to, or even in the absence of, cytochrome, the cells of aerobic organisms contain protohæmatin ; (b) that protohæmatin and corresponding hæmochromogens during development in the same organism (insects) gradually give place to cytochrome ; and, finally, (c) that ordinary protohæmochromogen (pyridine, nicotine, etc.) by successive oxidation and reduction can be transformed into substances displaying an absorption spectrum similar to that of cytochrome.

5. Under the action of hydrochloric acid, in the presence of a reducer such as  $\text{SO}_2$ , cytochrome *c* gives another porphyrin which can be considered as the unmodified *porphyrin c* of this compound. The position of the absorption bands of this porphyrin occupy an intermediate position between those of hæmato- and protoporphyrin, lying, however, nearer those of the former porphyrin. This porphyrin when recombined with Fe and a nitrogen compound such as pyridine gives a hæmochromogen indistinguishable from that of component *c* of cytochrome dissolved in aqueous pyridine. This unmodified porphyrin is insoluble either in ether and acetic acid or in chloroform, and its absorption spectrum can be measured either in acid or in pyridine. The porphyrin *c* on long standing in HCl in the presence of  $\text{SO}_2$  gradually becomes transformed into ordinary hæmatoporphyrin.

Our thanks are due to the Medical Research Council for defraying the expenses of this investigation.

## VI. REFERENCES.

- Bois, E. (1927). 'Recherches spectrochimiques sur quelques Porphyrins animales et sur les Combinaisons de L'Hématoporphyrine avec le Fer' (Fribourg, Switzerland).  
Hill, R., and Holden, H. F. (1926). 'Biochem. J.,' vol. 20, p. 1326.  
Fischer, H., and Linder, F. (1925). 'Z. Physiol. Chem.,' vol. 142, p. 141.  
Fischer, H., and Pützer, B. (1926). 'Z. Physiol. Chem.,' vol. 154, p. 50.  
Keilin, D. (1925). 'Proc. Roy. Soc.,' B, vol. 98, p. 312.  
Keilin, D. (1926). 'Proc. Roy. Soc.,' B, vol. 100, p. 129.  
Keilin, D. (1930). 'Proc. Roy. Soc.,' B, vol. 106, p. 418.  
Willstätter, R., and Fischer, M. (1913). 'Z. Physiol. Chem.,' vol. 87, p. 463.

*The Differential Action of X-Rays on Tissue, Growth and Vitality.—  
Part II. The Biological Reaction to X-radiation in Relation to  
a Phenomenon termed Antagonism.\**

By WARNFORD MOPPETT, M.D., Ch.M. (Sydney).

(Communicated by J. T. Wilson, F.R.S.—Received November 1, 1929.)

From the Biophysical Laboratories, the Department of Physics, the University of Sydney.

*Introduction.*

A selective action was observed when the allantoic membrane of the embryo chick was exposed to homogeneous X-radiation of different wave-length obtained by crystal diffraction (Moppett, 1929).

In the same communication a phenomenon termed antagonism, which implies a neutralisation of the effects of different components of mixed radiation was deduced from the sensitivity of the tissue to homogeneous radiation and its relative resistance to mixed radiation. The rays "reflected" from the crystal produced a visible change in about the same period of time as the "direct" rays obtained by removing the crystal. The relative intensities were found to be approximately 1/1000 by a photographic method and this ratio represents the relative potency of the two types of radiation under the experimental conditions described in the earlier paper. Antagonism appears to be a very striking phenomenon and it demands the condition that action must be in some respects selective so that the two factors may be considered together as a dual phenomenon.

When definite tissue changes are produced by the method of crystal diffraction, it is necessary to assume the presence of this dual phenomenon, since the energy available is exceedingly small compared with that ordinarily required to affect a tissue. The present paper contains experimental data which demonstrate the neutralising action between different wave-lengths which has been termed antagonism.

*Apparatus.*

Homogeneous radiation implies here a frequency band whose extent is determined by the finite size and disposition of the spectrometer slits and

\* This work has been carried out under the control of the Cancer Research Committee of the University of Sydney with the aid of the Cancer Research and Treatment Fund.

source of radiation. The earlier experiments were carried out by means of a Bragg spectrometer by Pye & Co., arranged as shown in fig. 1. The divergence

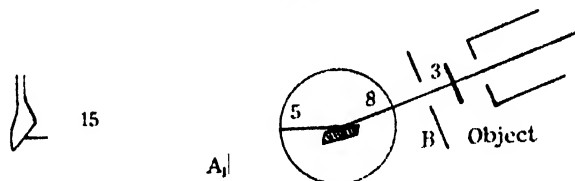


FIG. 1.

of the beam was controlled by slit A which was usually set at 1 mm., slits A and B being widely open. The projection of the focal spot on a plane at right angles to the axis of the spectrometer was 2 mm. wide so that the total divergence was 1/50. This value represents the extreme frequency band associated with each experiment and in a comparison of two different regions of the spectrum the degree of resolution attained. The wave-lengths calculated from the formula  $\lambda = 2d \sin \theta$  are therefore mean wave-lengths. A reference to fig. 2 will show that slit A controls the degree of overlap or homogeneity

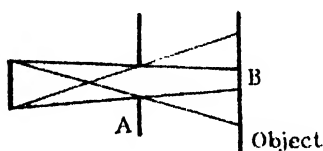


FIG. 2.

of the central part of the reflected beam and the area of object irradiated as well as the extreme frequency range. When slit A is opened an observer at B will see a larger portion of the focal spot until the whole is visible so that the energy per unit area in the central portion of

the reflected beam is also a function of the slit width.

The later work embodied in the graph (fig. 4) was carried out by means of a "lathe bed" spectrometer specially designed for biological work using a wave-length of 0.27 Å. and an angular divergence of 1/150. This apparatus was energised by means of a water-cooled Coolidge deep therapy tube and a constant potential generator by Gaiffe Gallot. A kilovoltmeter reading of 55 was maintained and the milliamperage was varied from 1.5 to 15, the output remaining perfectly constant over the periods of exposure. It is reasonable to assume under the above conditions that the output of any particular frequency is proportional to the milliammeter reading at least to a first approximation.

#### *A Standard Biological Reaction.*

The following experiments have been carried out with reference to a biological reaction which takes place when the Pye spectrometer is set to give a mean wave-length of 0.53 Å. This radiation lies in a convenient region of the

spectrum and under the conditions described, produces an atrophic reaction when the exposure exceeds  $1\frac{1}{2}$  hours, a hypertrophic reaction between  $\frac{1}{2}$  and  $1\frac{1}{2}$  hours and no visible change with an exposure of less than  $\frac{1}{2}$  hour. The three states are expressed in the following tables by the letters, A, H and O respectively, and a transition from one to another represents a considerable variation of effect in terms of the values given. Typical experiments are quoted and their reliability is indicated by the ratio of consistent to inconsistent results, an abnormal response being observed about once in every 33 experiments (Moppett, 1929). All experiments were carried out in pairs or small groups so that one should be a control on another, and investigations of a supposed law extended over a considerable period of time to guard against possible seasonal variations.

(1) *The Effective Frequency Band.*—The divergence of  $1/50$  with a 1-mm. slit (A, fig. 1) appears to be an optimum value since any considerable variation diminishes the degree of reaction. In the following table, experiment No. 181 is given as a standard of comparison, and experiments Nos. 36 and 29 show a diminished reaction in association with a narrow and wide slit respectively. The incident beam is restricted by slit B so that the area irradiated may be the same in each case and the milliamperage is weighted against the conclusion. The reliability factors indicate pairs of experiments in which the supposed law was tested under a variety of different conditions. In the case of No. 29 three experiments were found to be invalid owing to a small aperture in a protecting lead box and later work has rendered repetition unnecessary although the reliability factor is nominally  $3/6$ .

Experiment No. 73 illustrates the fact which was fully discussed in the earlier paper that with narrow slits there is a definite shift of the region of maximal effect to the soft ray side.

Table 1.

Number of experiment.	Mean wave-length.	Slit width.	Time of exposure.	Milli-amps.	Incidence on specimen.	Remarks.	Results.	Reliability.
181	0.53	mm. 1	hours 2	4	Normal	Slit B 3 mm.	A+	58/60
36	0.53	0.5	2	5	"	"	H	10/11
29	0.53	5	2	5	"	"	H	3/6
73	0.58	0.5	2	5	"	"	A	3/4
822	0.48	1	2	4	60°	"	H	{ Observation well-established.
862	0.53	1	2	4	60°	"	A+	

Experiments S 22 and S 52 illustrate the sharp definition of selective action particularly on the hard ray side which is rather surprising in view of the wide angular divergence used. The nature of the reaction is determined almost exclusively by the mean incident frequency, a phenomenon which was observed at every region of the spectrum.

#### *Discussion.*

A possible explanation of the above observations is furnished by supposing that the reaction under consideration is associated with a definite frequency band which corresponds roughly with the optimum divergence of  $1/50$ . A reference to the earlier paper will show that the existence of a similar frequency band for all reactions was deduced from the general form of the graph and in the region beyond  $1 \text{ \AA}$ . similar effects were observed at more than one angular setting of the crystal. The term mean wave-length may therefore be extended from the physical application to designate the mean wave-length of a reaction. The sharp definition of the selective action also suggests that all frequencies beyond the effective band tend to neutralise the reaction. If the effective band were very narrow one would expect that the increased homogeneity obtained with narrow slits would more than compensate for the loss of energy per unit area (fig. 2).

This is not so although the position is complicated by the peculiar shift of the reaction towards the soft ray side with narrow slits. An accurate determination of the mean frequency and frequency range of a reaction is rendered difficult by the undue prolongation of exposure when the divergence and area of object irradiated is very small and a prolonged investigation will be necessary to settle these important questions.

(2) *Neutralisation by Frequencies beyond the Effective Range.*—A number of experimental facts can be readily explained on the assumption that one frequency may neutralise another in its biological action. Experiment 4, Table II, illustrates the fact fully discussed in the earlier paper that the radiation scattered from egg shell may prevent a reaction from taking place. Experiments (6.8.26), (10.6.26) and (30.7.26) illustrate the fact that reactions could not be obtained at one period in a new copper box which contained the biological specimen unless a very long exposure was given. Photographic evidence showed that direct rays were striking the box and exciting a comparatively large amount of scattered and fluorescent radiation. A lead shield was fitted and consistent reactions were obtained and this was later removed and replaced again as a check. The remaining experiments in Table II illustrate

a determination of the threshold dose for mixed radiation with various generators. The reaction represented by R appears to be related to the energy curve since the milliamperage and potential were of the same order throughout. With a coil and mercury break the threshold dose lies between 1 and 2 hours, the three short exposures excluding a zone phenomenon or the production of a reaction with a small dose and its suppression with a large dose.

With a transformer the dose was between  $\frac{1}{4}$  to  $\frac{1}{2}$  hour and with a constant potential generator between  $\frac{3}{4}$  and 1 hour. The latter radiation would represent the closest approach to homogeneity but an infinite variety of low frequencies is still present. The threshold dose after crystal diffraction certainly did not show such a variation and the results can scarcely be explained in terms of intensity.

Table II.

Number of experiment.	Mean wave-length.	Slit width.	Time of exposure.	Milli-amps.	Incidence on specimen.	Remarks.	Result.	Reliability.
4	0.53	mm. 2	hours 1 hr.	5	Normal	Scattering from egg shell	O	3.3
(6.8.26)	0.53	2 approx.	1 $\frac{1}{2}$ hrs.	5	..	Scattering from copper	O	5.5
(10.6.26)	0.53	2 ..	3 ..	5	..	..	H	Single
(30.7.26)	0.53	2 ..	1 $\frac{1}{2}$ ..	5	..	Lead shield	H	6.6
	(3 at 0.84)							
	Direct rays	2 ..	2 mins.	4	..	Coil and mercury break. 65 KV.	O	—
			3 ..					
			3 ..					
			10 ..					
			18 ..					
			60 ..					
227	..	2 ..	2 hrs.	4	..	..	R	Single
	..	2 ..	$\frac{1}{4}$ hr.	4	..	Transformer 65 KV.	O	3.3
224	..	2 ..	$\frac{1}{4}$ ..	4	..	..	R	3 + $\frac{1}{4}$
	..	Lathe bed spectro-motor	30 mins.	4	..	Constant potential. 65 KV.	O	—
			35 ..					
			40 ..					
			45 ..					
			50 ..					
			55 ..	4	..	..	R	—
			60 ..					

The apparatus shown in fig. 3 was constructed in order to obtain further evidence of the neutralisation between different frequencies. Two crystals were provided with a lateral movement and the wave-length was estimated from the formula  $d/\lambda = \tan \theta$ . The biological specimen was placed at the point of coincidence and received a simultaneous and approximately equal

dose of the two radiations. One crystal was fixed to give a mean wave-length of 0.53 Å. and the exposure was arranged to produce atrophy, four experiments

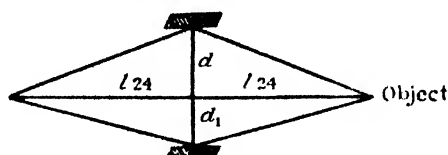


FIG. 3.

being performed. This work is by no means complete, but it was observed in 11 cases out of 15 that the reaction was suppressed or reduced to lower degree by the admixture of any other radiation. It was also observed from 5 experiments the low frequencies were more effective in producing neutralisation than the high ones. Contrary to expectation a second atrophy-producing frequency produced neutralisation rather than reinforcement in 3 consistent experiments. According to practice the time of exposure was varied somewhat, but experiments showing an average response are given in Table III, the letters HA representing an easily recognised stage between hypertrophy and atrophy.

Table III.

Wave lengths.	Nature of added frequency.	Result.	Reliability.
0.5 control		A+	4/4
0.5 + 0.3	Ineffective hard radiation	A	
0.5 + 0.6	Specific hypertrophy	O	
0.5 + 0.7	Ineffective soft radiation	HA	11/15
0.5 + 0.8	Second atrophic reaction	HA	
0.5 + 0.9	Specific hypertrophy	HA	

### Discussion.

The data given in Tables II and III afford ample experimental evidence in support of the phenomenon which has been termed antagonism. Neutralisation of a biological change does not appear to be the result of admixture with any particular frequency band, but there is much evidence, though hardly amounting to proof, that the low frequencies, particularly those near the effective band, are most potent in this respect. This is in accord with the shift of a reaction with increased resolution. The addition of high frequencies is similar to the addition of higher order spectra. In the investigation of selective action (1929) a fallacy was suggested owing to the higher order spectra of tungsten, the target element. In all such work the potential was arranged

so that the K lines of tungsten would not be excited, but it appears that the addition of higher order spectra would diminish rather than increase a reaction.

In the case of mixed radiation one may assume that the action of one arbitrarily chosen frequency band is nullified to a greater or less extent by other frequencies present. In this manner different energy curves may furnish different threshold doses. The term balanced radiation may be applied to one or many types of mixed radiation which show a maximum threshold dose. It is possible that such a radiation would never affect a tissue or if any reaction was observed one might suppose a different phenomenon, a breakdown to a continued bombardment. Mixed radiation with a relatively low threshold dose may be regarded as unbalanced radiation on the supposition that there is a residual component force in one direction which might be expected to possess properties very similar to those of homogeneous radiation.

(3) *Prior Action.*—It was observed that an atrophic reaction was prevented by a prior exposure of 10 minutes to the direct rays obtained by removing the crystal. The observation was confirmed by tilting a specimen so that the radiations would not overlap and a small area of atrophy was obtained corresponding to one corner of the area irradiated. Each experiment was associated with a control in which the mixed radiation was applied after the homogeneous radiation and a normal reaction resulted. These results are summarised in Table IV and the fact that the mixed radiation produces no visible change is illustrated by experiment No. 300. An exposure of 4 minutes or less to direct radiation did not produce neutralisation and an exposure of 5 minutes was apparently on the borderline.

Table IV.

Number of experiment.	Mean wave-length.	Slit width.	Time of exposure.	Milli-amps.	Incidence on specimen.	Remarks.	Result.	Reliability.
306	0.53	mm. 1	hours 2	4	60	Prior exposure 10 min. direct rays	O	7/7
305	0.53	1	2	4	60	Subsequent exposure 10 min. direct rays	A	7.8
300	Direct rays	1	1	4	60	—	O	—
296	0.53	1	2	4	60	Prior exposure 5 min. direct rays	O	5.7
192	0.53	1	2	4	60	Prior exposure, 4, 3, 2, 1 min. direct rays	A	4.4



A series of experiments was made with the constant potential apparatus in order to amplify the above observations. All the experiments received a similar exposure of  $\frac{1}{2}$  hour and the results were recorded on a graph, fig. 4, hypertrophy being represented by the shaded circles and no reaction by the open circles. Milliampereage is plotted in a vertical direction and the left-hand portion of the graph is devoted to a determination of the threshold dose. The raising of the threshold dose by means of a prior application of mixed radiation is indicated along the right-hand portion of the graph. Ten minutes' exposure was given at 65 KV. and various millimeter readings which are indicated along the baseline and the results are connected by a smooth curve.

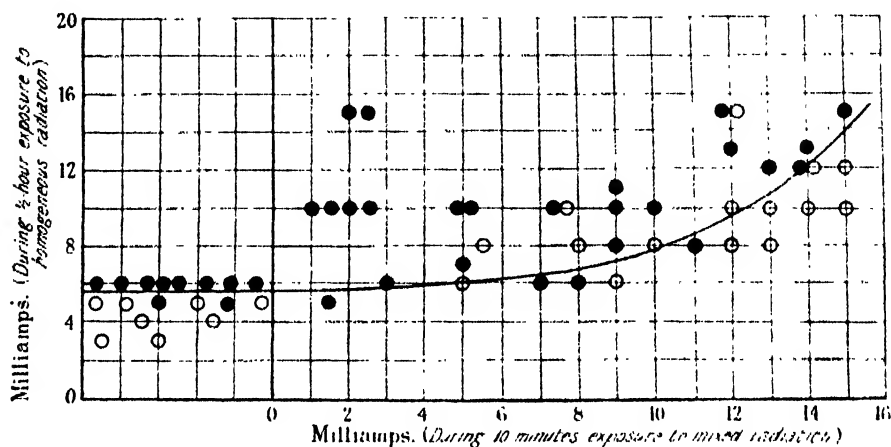


FIG. 4.

### Discussion.

The phenomenon of prior action suggests that all manifestations of antagonism may be considered in similar terms, that is the ineffective radiation first determines the state of the tissue. It is also to be observed that mixed or balanced radiation may produce a change of state without any visible reaction. A possible explanation is furnished by the production of ionised atoms, whose effects may neutralise one another in the case of balanced radiation.

It will be seen from Table IV that the neutralising radiation must be approximately 80 times greater than the effective radiation, using the approximate ratio of 1000 to 1. The experiments depicted in fig. 4 were carried out mainly to obtain more information on the point. It is not possible to determine the exact form of the curve, but the data suggest a hyperbola which would be in accord with a progressive neutralisation between opposing forces. It will be

observed that the experiments on the left-hand side of the graph were very consistent whilst there were considerable variations along the curve! The disposition of results indicates that it was expected that the curve would bend upwards more quickly, but the neutralising radiation may have been unsuitable. If the curve were continued matters would be complicated by the development of a reaction due to the mixed radiation. Ideally one might employ balanced radiation for neutralisation and different points on the curve would represent different types of unbalanced radiation approaching asymptotically a condition of balance.

#### *Conclusions.*

The present paper sets out to confirm experimentally a phenomenon termed antagonism which implies a neutralisation between different frequencies in respect of their biological action. Some attempt has been made to define an effective frequency band and in all instances the addition of frequencies outside the band tends to diminish or prevent biological change. The last part of the paper is devoted to a special aspect of the case in which antagonism is associated with prior action. The fact that mixed radiation produces a refractory state without any visible biological change suggests that both the action and its neutralisation are associated with preliminary changes in the atom which may be regarded as some form of ionisation.

#### REFERENCE.

Moppett (1929). 'Proc. Roy Soc.,' B, vol. 105, p. 403.

*The Differential Action of X-Rays on Tissue Growth and Vitality.—  
Part III. The Biological Reaction to X-Radiation in Relation to  
the Area of Tissue Irradiated.\**

By WARNFORD MOPPETT, M.D., Ch.M.

(Communicated by J. T. Wilson, F.R.S.—Received November 1, 1929.)

From the Biophysical Laboratories, the Department of Physics, the University of Sydney.

[PLATE 19.]

The allantoic membrane of the embryo chick was exposed to homogeneous X-radiation obtained by crystal diffraction and a selective action was observed (Moppett, 1929). A particular wave-length  $0.53 \text{ \AA}$ . produced a hypertrophic reaction with an exposure of  $\frac{1}{2}$  hour and an atrophic reaction in  $1\frac{1}{4}$  hours and this was adopted as a standard in the investigation of problems other than selective action (Moppett, 1929 and 1930).

*The Effect of Irradiating a Large or Small Area.*

A variation in effect with the area irradiated was suggested because at an early stage reactions were readily produced with an imperfect crystal and it was found that the results could be imitated by placing the specimen at an oblique angle to the incident radiation. It appears that the imperfect crystal was "convex," spreading the rays out to a greater extent than usual and various aspects of the problem are illustrated by the following typical experiments.

Hypertrophy is represented by H and atrophy by A, and the transition from one to the other by placing the specimen at an oblique angle is illustrated by experiments 35 and 318 in which all other conditions are similar. In other cases the transition might be from no reaction to H, but there was always a considerable reinforcement and the general observation is well established as the results of some years' investigation. In experiment 218 atrophy was produced over an area of  $1 \times 4 \text{ cm.}$  at grazing incidence and no limit to the above reinforcement has been found. In order to obtain further information,

\* This work was carried out under the control of the Cancer Research Committee of the University of Sydney and with the aid of the Cancer Research and Treatment Fund.





III



experiment No. 111 was placed at an oblique angle, but the area irradiated was restricted (by a slit) and no reinforcement was observed. This result was obtained in two consistent experiments as indicated by the factor 2/2.

In experiment 96 a normal incidence was used similar to experiment 35, but the specimen was withdrawn to approximately twice the distance from the target and the reaction was A+ (R2). The area irradiated was multiplied by 4 in the above case and the general observation is well established within these limits.

In the case of 62 a large area was covered by moving the object continuously during exposure, and no reinforcement was observed in two consistent experiments, although one specimen showed two hypertrophic reactions (Plate 19, fig. 7).

The remaining experiments were carried out with a constant potential generator on the assumption that the intensity of any radiation should be proportional to the milliamperage. The threshold dose for hypertrophy at a wave-length of 0.27 Å. was accurately determined in terms of a current of 6 mA in connection with another investigation (Part II). The slit height was then reduced so that 6/10 of the original area should be irradiated, and in accordance with experiment No. 1067 the threshold dose was represented by 15 mA, the ratio being approximately inversely as the square of the area.

Table I.

Number of experiment.	Mean wave-length.	Slit A width.	Time of exposure.	Milli-amps.	Incidence on specimen.	Remarks.	Result.	Reliability.
	Å	mm.	hours					
35	0.53	1.5	2	4	Normal		H	Well-established
318	0.53	1.5	2	4	60°		A +	
218	0.53	2	2	4	Grazing		A + + +	
111	0.53	2	2	4	60°		H	Single
96	0.53	1.5	2	4	Normal	Slit B, 1 mm. wide Distant 40 cm. from crystal	A :	2/2
62	0.53	2	2	5	..	Specimen was moved 1 mm. each 1/2 hour	H (double)	Well-established
995	0.27	—	1/2	6	..	Constant potential, full area	H	2/2
1066	0.27	—	1/2	12	..	Constant potential, area 6/10	.	8/8
1067	0.27	—	1/2	15	..	..	H	4/4

*Discussion.*

When the allantoic membrane is placed at an angle  $\phi$  to the incident radiation, the energy per unit area is reduced to  $\cos \phi$  times the energy at normal incidence. The allantoic membrane has an average thickness of 0.02 cm., and the total  $x$  absorption is very small so that cells in different parts will absorb nearly the same amount of energy whether the incidence is normal or oblique.

The above consideration will not account for the raising of degree of reaction or the reinforcement observed when the distance from the target is increased. In the case of an atrophic reaction one may postulate a greater interference with nutrition, since circulation proceeds from the periphery of the area under consideration.

In a discussion of the development of an atrophic reaction, it was supposed that all types of tissue are rapidly killed by the action aided by an interference with circulation due to a simultaneous injury to the blood vessels (Moppett, 1929).

The figures marked A, B and C (Plate 19) illustrate this. In A, a borderline stage between atrophy and hypertrophy is depicted as a commencing degeneration in the centre of a hypertrophied mass. In B, a central island of hypertrophied tissue remains, but the nutrition is cut off by the surrounding atrophy. Fig. C (Plate 19) is a high-power view showing a preliminary hypertrophy of endothelial cells in an area where atrophy is almost complete. An interference with nutrition will not entirely account for the state of affairs depicted in A, or in the case where a hypertrophic reaction is obtained at oblique incidence and no change at normal incidence. One must assume another factor, a summation of stimuli from adjacent parts and the relation of the threshold dose to the reciprocal of the square of the area irradiated suggests a force obeying some definite law.

*General Aspects of the Area Factor.*

It will be convenient to review the more general aspect of the above conclusions in terms of certain typical cases which represent the result of a very extensive observation, although it must be mentioned that the obtaining of "copper plate" specimens for reproduction is by no means easy.

1. A dose which is just above the threshold for hypertrophy produces a very small reaction, which is approximately circular and lies at what may be termed the "centre of gravity" of the irradiated area. The rectangle in

fig. 1 (Plate 19) shows the approximate size and form of a cross section of the incident beam of rays, and the dose is represented by the number of vertical lines per unit distance, while the reaction is shown in half-tone, and an actual photograph is also reproduced. In accordance with the supposition already made one may regard the reaction as due to a summation of stimuli from all parts of the irradiated area.

2 and 3. The next figure illustrates the reinforced action obtained with oblique incidence and an actual photograph is also reproduced, while fig. 3 (Plate 19) depicts the overflow to a region of inadequate dosage which was discussed in an earlier paper (Moppett, 1929).

4. If a normal incidence is used and the dose is increased, the area of membrane showing a visible reaction increases and may overflow beyond the irradiated area. This is a converse to the increased reaction which is associated with a larger area irradiated.

5. In fig. 5 (Plate 19) two discrete areas separated by a gap of 2 mm. were irradiated and the reaction was situated at the "centre of gravity" of the disturbance, although this region received no radiation apart from scattering. Threesimilar experiments were performed and the observation is well established by early unsuccessful attempts to produce a geometrical figure on the above lines.

6. If a sufficiently large gap is provided, discrete reactions can be obtained, and a photograph is given of an intermediate stage showing a dumb-bell shaped reaction. When small circular apertures were used, discrete reactions could not be obtained, but the threshold dose was found to vary inversely as the square of the distance between centres for the following values 3.5, 4.5, 5.5 and 6.5 mm.

The above facts preclude the production of geometrical figures on the scale of magnitude available.

Fig. 7 shows a patchy reaction which is occasionally observed when a comparatively large area receives a dose near the threshold value. There are presumably small variations in intensity or sensitivity over the area covered, and the double reaction recorded in Table I may be explained on this basis.

#### *Experiments with Mixed Radiation on Mammalian Tissues.*

The above laws were tested by means of mixed radiation on the assumption that it was unbalanced radiation (Moppett, 1930), having a component of force similar to that of homogeneous radiation. The skin covering the back of a rat was immobilised in a wooden frame by means of four stitches, and



the hair was removed by sodium sulphide. A lead mask was used with four square apertures of various sizes, and the various doses tabulated below were given by means of a constant potential apparatus, the results being observed after an interval of 7 days.

Table II.

Dose.	Apertures.		
	0.25 <sup>2</sup> .	0.5 <sup>2</sup> .	2 <sup>2</sup> .
Milliamp. minutes			
200			E slight
250			E
300			E
450		E	E
600		E	Covered
450	}	Killed when large aperture included.	
600			

It will be seen that the erythema dose increased as the aperture diminished slowly at first and then more rapidly, and no doubt an inverse square law could be demonstrated if fixation was sufficient to justify the use of very small apertures.

To test the phenomenon of fusion a mask was prepared with 5 pairs of circular apertures, 0.25 cm. diameter and 3.5, 4.5, 5.5, 6.5 and 7.5 cm. apart. The discrete erythematous areas were readily identified and a connecting link of reddened skin was observed as indicated by the letter F in the following table.

Table III.

Dose.	Apertures.					(Fusion distance) <sup>2</sup> .
	3.5.	4.5.	5.5.	6.5.	7.5.	
Milliamp. minutes						
675	F	—	—	—	—	1.4
1080	F	F	—	—	—	1.3
1600	F	F	F	—	—	1.3

### Conclusions.

There is ample evidence that the threshold dose for a biological reaction varies as the area irradiated both when the allantoic membrane is exposed to

homogeneous radiation and the skin of the rat to mixed radiation. This result, together with the phenomenon of fusion, may be explained in part by a summation of stimuli from distant parts in such a manner that an inverse square law is manifested when the area irradiated becomes small. It is to be observed that distant regions may be affected even if an area under consideration shows no visible change. The mechanism of remote action is therefore not concerned with the production of visible changes, such as hypertrophy and atrophy, but with an intermediate stage which follows the absorption of quanta and presumably the production of ionised atoms. The present discussion strengthens the supposition made in connection with prior action that the biological change is due to the production of ionised atoms (Moppett, 1930). It is reasonable to suppose that the threshold dose would become enormously great if a very small area involving very few atoms was affected and the law may presumably be extended to three dimensions.

Prof. V. A. Bailey has kindly pointed out the following consequence of the supposition that in the production of a particular biological change the following law holds,  $IA^2 = \text{constant}$ , when  $I$  is the intensity of the radiation and  $A$  the area.

If  $N$  represents the number of ions (of one sign) produced in the area  $A$ ,  $N$  is proportional to  $IA$  and the above equation becomes  $NA = \text{constant}$ .

In a similar manner, if there are  $N_1$  other factors (ions of opposite sign, cells, etc.) involved in the area then  $N_1$  is proportional to  $A$  and so  $NN_1 = \text{constant}$ . The inverse square law may, therefore, indicate that the reaction requires the co-operation of two different factors (ions, etc.) to produce a definite number of transactions each involving one factor of each kind.

One must recognise a purely biological overflow in the general rounding off of inflammatory processes, but in the present instances it appears best to regard gross biological changes such as hypertrophy and atrophy as distinct phenomena which somewhat complicate the interpretation of effects depending on the atom.

#### REFERENCES.

- Moppett (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 403.  
Moppett (1930). 'Proc. Roy. Soc.,' B, vol. 107, p. 293.

*The Differential Action of X-Rays on Tissue, Growth and Vitality.—  
Part IV.—The Biological Reaction to X-Radiation in Relation  
to Time.\**

By WARNFORD MOPPETT, M.D., Ch.M. (Sydney).

(Communicated by J. T. Wilson, F.R.S.—Received November 1, 1929.)

From the Department of Physics, the University of Sydney.

*Preliminary Experiments.*

In the investigation of a selective action of homogeneous X-radiation on the allantoic membrane of the chick (Moppett, 1929), a constant time of exposure was adopted since one cannot assume that the "time factor" is expressed by a linear function. It was observed at an early stage that a considerable fluctuation in current supply did not materially alter the biological reaction if the normal time of exposure were given. An experimental investigation was carried out using a wave-length of 0.5 Å. which produces a hypertrophic reaction with an exposure of  $\frac{1}{2}$  hour and an atrophic reaction in  $1\frac{1}{2}$  hours, when other conditions are similar. The reaction shows up abruptly when the above values are exceeded, suggesting the breakdown of a process of repair and the phenomenon is aptly described by the term threshold dose.

Typical experiments are given in the following table (1) and the results are represented by the letter A for atrophy, H for hypertrophy, and HA for an intermediate stage. Experiment 147 may be taken as a standard in which a certain quantity of energy is given over a period of 1 hour, the reaction being HA. In experiment 135, approximately the same dose was given over a period of 2 hours and the reaction was increased to A+. The reliability factor 5/5 indicates that five consistent pairs of experiments showed an increased reaction in association with a longer time of exposure. Experiment 142 illustrates the fact that an increased reaction is obtained even if the exposure is given intermittently over a long period of time.

The above variation is opposite in sign to what may be termed a repair factor which is illustrated by experiments 306 and 308. A reaction to homo-

\* This work was carried out under the control of the Cancer Research Committee of the University of Sydney and with the aid of the Cancer Research and Treatment Fund.

geneous radiation may be prevented by an adequate dose of non-effective mixed radiation given immediately before the effective radiation (Moppett, 1930.) If an interval of 48 hours is made between the two applications a normal reaction results, indicating that the protecting action of the mixed radiation has been annulled.

Table I.

Number of experiment.	Wave-length.	Slit width.	Time of exposure.	Milli-amps.	Incidence on specimen.	Remarks.	Result.	Reliability.
	Å.	mm.	hours					
147	0.5	1	1	4	60	—	HA	5/5
135	0.5	1	2	2	60	—	A+	
142	0.5	1	2	4	60	Interrupted 10 min. on, 10 min. off	A+	
306	0.5	1	2	4	60	Prior dose 10 min. direct rays	No reaction	7/8
308	0.5	1	2	4	60	Prior dose 15 min. direct rays 48 hrs. before homogeneous rays	A+	2/2

### *Confirmatory Experiments.*

In order to confirm the above indications, a series of experiments was made with a constant potential apparatus by Gaiffe Gallot on the assumption that the output of any frequency should be roughly proportional to the millimeter reading. A wave-length of 0.27 Å. and a potential of 65 KV. were used throughout and the results are expressed graphically in fig. 1 where the open circles represent no reaction and the shaded circles a hypertrophic reaction. The product of milliamperage and time is plotted in a vertical direction and time along the baseline and the results for  $\frac{1}{2}$  hour exposure shown by double circles represent an accurate determination of the threshold dose in connection with another investigation (Part II). The milliamperage could not be reduced sufficiently to obtain no reaction when the exposure exceeded 1 hour, so a filter of aluminium 12 mm. thick was used to give a  $\frac{1}{4}$ -value intensity and the product of time and milliamperage was divided by 4 in plotting these results.

The threshold dose is represented on the graph by a line which passes between the open and shaded circles and in this region one obtains an equal number of reactions and blanks owing to variations covered by the term idiosyncrasy. It may be assumed that a greater dose will give a reaction and

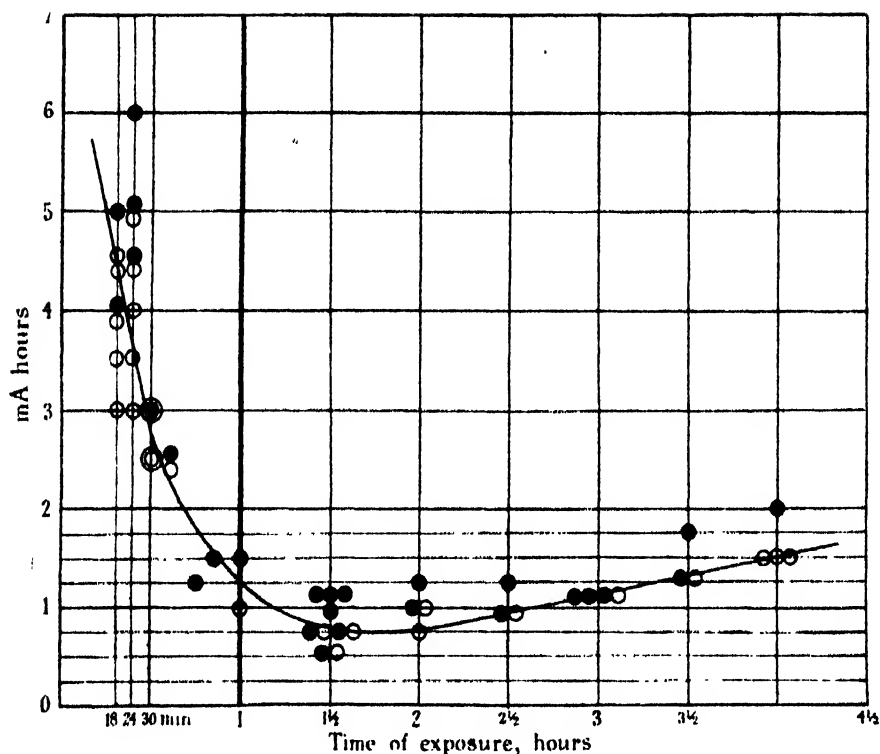


FIG. 1.

a smaller dose no reaction, for example on the right-hand portion of the smooth curve.

### *Investigations of the Action of Mixed Radiation on Mouse Skin.*

The investigation was extended to the skin of the mouse by the use of unfiltered mixed radiation, which was regarded as unbalanced radiation (Part II.) The hair was removed with sodium sulphide and the result was observed in 7 days and plotted in a similar manner in fig. 2, where the closed circles represent an erythema which is apparent as a region of slight desquamation in borderline cases. The skin was immobilised by four stitches beneath a lead shield with an aperture 0.4 cm. square, a value which was chosen so that the threshold dose, which varies with the area irradiated, should suit the characteristics of the apparatus (Moppett, 1930a).

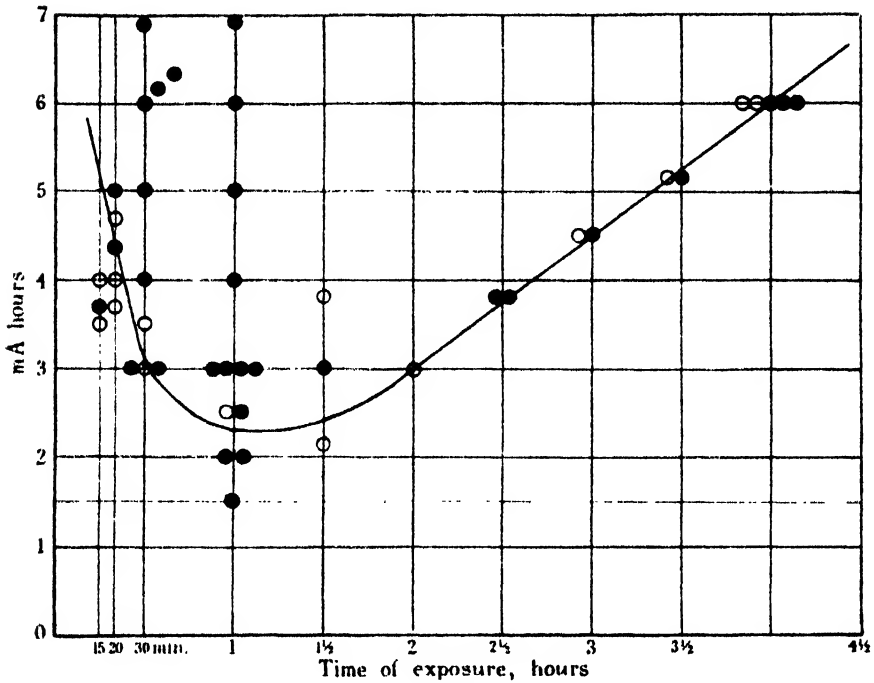


FIG. 2.

### *Discussion.*

The "time" curve presents a minimum value at about 1 to 1½ hours both when the allantoic membrane is exposed to homogeneous radiation and the skin of the mouse to mixed radiation. This confirms the experiments in Table I, but there a minimum is indicated at 2 hours or more, which may be a characteristic of the wave-length used. The two upward branches represent a "positive factor" when  $t$  is small and a "repair factor" when  $t$  is large. The explanation may be considered in terms of the mechanism of development of the reaction which was divided into a physical stage, a chemical stage, involving the ionised atoms and a final biological stage.

The positive factor may represent a physical phenomenon implying that when the rate of absorption of energy is small, the ordinary laws of probability do not hold and the atom can only absorb quanta when it is in a certain state which recurs cyclically. The form of the curves (fig. 4, Part II) suggest that the threshold dose approaches infinity as " $t$ " approaches zero. The above phenomenon may be related to the biological stage implying a cyclical receptive state in the living cell, but this appears less likely. The repair factor is also capable of an explanation as a physical or biological phenomenon. In the

case of the allantoic membrane, it may be expressed as the equivalent of a current of  $-\frac{1}{2}$  milliamp. under the experimental conditions of this investigation (Parts I, II, III). The most likely explanation appears to be that of a return of the ionised atom to a normal state which must hold in experiments 306 and 308, since the mixed radiation produces no biological change.

In accordance with the supposition already made, one may assume that the ionisation is of some special or restricted nature, which is covered by the non-committal term "saturated receptor."

One may also assume a purely biological process of repair to exist which may complicate the interpretation of changes in the atom. The possibility of cumulative action for which there is much evidence in X-ray literature may be explained as an ultimate breakdown of the biological aspect of repair.

The curves may be approximately represented by the equation

$$y = k/t + (a + \beta)t$$

where  $y$  is the threshold dose and  $t$  the time of exposure and  $k$  and  $(a + \beta)$  are constants.

The repair factor is represented as the sum of two constants of which  $a$  is related to the atom and  $\beta$  to a biological process of repair. The repair factor for mouse skin has a larger value than that for the allantoic membrane and where similar atoms are concerned one may assume that  $a$  has the same value, but  $\beta$  is larger representing a more effective repair in the more highly organised tissue. This would indicate that the resistance of mouse skin both to mixed and homogeneous radiation is much greater than that of the allantoic membrane although in all probability the ultimate phenomenon is similar.

#### REFERENCES.

- Mopett (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 403.  
Mopett (1930). 'Proc. Roy. Soc.,' B, vol. 107, p. 293.  
Mopett (1930a). 'Proc. Roy. Soc.,' B, vol. 107, p. 302.

*The Mechanism of Ciliary Movement.—VI. Photographic and Stroboscopic Analysis of Ciliary Movement.*

By J. GRAY, F.R.S. (from the Zoological Laboratory, Cambridge).

(Received October 1, 1930.)

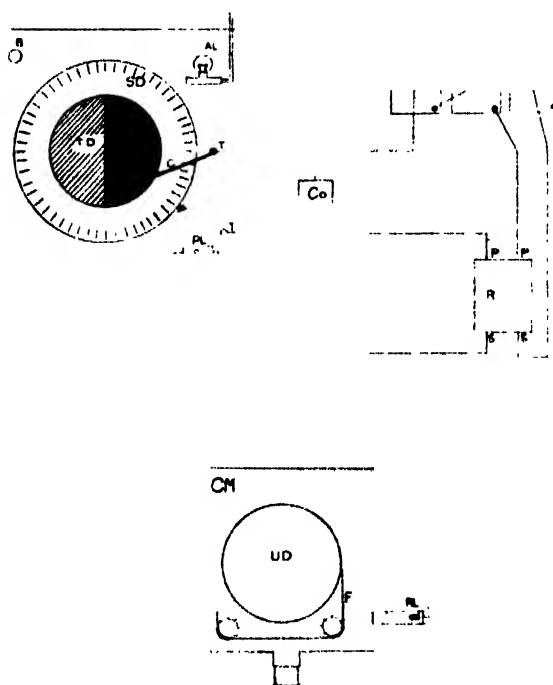
[PLATES 20-22.]

When viewed under a suitable power of the microscope a normally moving cilium seldom, if ever, yields the impression of a well-defined image to the human eye. The blurred image, which is observed, is due to the idiosyncrasies of the eye rather than to the size or velocity of movement of the cilium. As explained elsewhere (Gray, 1928), the tip of an active cilium is seldom moving more rapidly than 3 inches a minute, but it is reversing the direction of its movement about 30 times per second. In order that the eye should register a clear image of a moving object, it is essential that the axis of the eye should move simultaneously in the direction of motion, for by so doing the image of the object is focussed on the same region of the retina for a requisite period of time. Since normal cilia reverse their direction of movement at a frequency far in excess of that which is possible for the axis of the human eye, a moving cilium registers, on the retina, a series of closely adjacent and super-imposed images which are responsible for the sensation of a blurred image. Hitherto the analysis of ciliary movement has been restricted to methods which are either concerned with the nett result of the activity of a ciliated epithelium or with the behaviour of individual cilia whose normal frequency has been artificially reduced to a very low level. By the use of a suitable cinematograph camera and a simple stroboscope this limitation of enquiry can be largely overcome. The first section of this paper deals with the development of the necessary technique and is followed by others which are concerned with specific problems of ciliary movement.

The form and duration of the two phases of a single beat of an individual cilium can only be recorded photographically if the period of time involved is large in comparison to the duration of a single cycle of the camera being used. If the period of complete ciliary beat is 0.1 second, the minimum frequency of the cinematograph camera must be of the order of 100 exposures per second. So far, frequencies of this order have proved impracticable, for



the apparatus available would not permit of frequencies higher than 24 per second. Fortunately, the normal duration of the beat of the large abfrontal cilia on the gills of *Mytilus edulis* is of the order of 0.5 second, and it has proved possible to take 12 photographs during this period. The apparatus used (Plate 21, fig. 1) is a modification of that supplied by Messrs. E. Leitz, and consists of an optical bench, microscope and camera mounted as one unit and suspended by vertical springs. As supplied by the makers, the apparatus does not record the frequency of the exposures and for this purpose two alternative methods have been employed. The first of these is illustrated diagrammatically in text-fig. 1. A small hole, L, is drilled in the base of an Ascania camera, and into it is fitted the lens of a 1-inch microscope objective to which is attached a



TEXT-FIG. 1.—Mechanism for recording the frequency of exposure of a cinematograph camera.

metal sleeve; inside the sleeve is fitted a small 4-volt endoscope lamp (RL) whose dimensions are approximately 2 mm. by 1 mm. By suitable adjustment, the image of the lamp can be focussed on the edge of the photographic film (F) as the latter passes over the continuously moving sprocket wheel, into the up-take drum (UD) of the camera. In the lamp circuit is placed the timing device,

TD. The latter consists of an induction motor fitted with a brake and carrying in place of the usual steel plate two smaller concentric discs. One of these (SD) is painted white and is divided into  $3^\circ$  sectors by a series of black lines. The other disc (TD) is composed of two halves, one of brass and the other of hard rubber. The endoscope lamp circuit is made through the body of the motor at T and the sliding contact C. The whole apparatus is enclosed in a wooden box fitted with a window and illuminated from the inside by an alternating lamp (AL) of 60 cycles. In order to calibrate the time marker, the motor is switched on and the brake adjusted until the black lines on the disc (SD) appear stationary. At this point the inner disc TD is revolving once per second, and the endoscope lamp circuit is closed for half a second and open for half a second; with a reliable 60-cycle current, the timing of the motor was remarkably constant. On developing a strip of film, alternate bands of light and dark are found on the edge of the negative and the length between the start of each dark band gives the length of film passing through the gate of the camera per second, and hence the interval between two successive photographs can readily be calculated. It is convenient to insert a relay (R) between the timing device and the lamp circuit, and to mount a pilot light (PL) in series with the endoscope lamp since the latter is not visible from the exterior of the camera. There is one objection to this type of time recorder: the small endoscope lamp takes a small but measurable time to glow on closing the current, so that lengths of film passing through the camera cannot be very accurately determined except over complete intervals of 1 second. This objection is eliminated in the second type of recorder.

In the second type of recorder, the principle of marking the edge of the film is the same, but the necessity of lighting and extinguishing the light is avoided by interrupting a beam of continuous light by an opaque shutter. This shutter is carried by the moving spring of an electromagnet. The magnet is excited four times a second by a suitable electric clock and by adjusting the aperture of the shutter, clear markings at quarter-second intervals are effected on the developed film.

Knowing the time interval between successive exposures, it is possible to reconstruct the time relationships of a single beat of a cilium or in certain cases the interval between successive beats. With the camera available it is not feasible to increase the number of exposures per second greatly in excess of 24. By using the slowly-beating abfrontal cilia of *Mytilus edulis*, a series of 10 photographs have been obtained of one beat (see Plate 20); alternatively the records can be expressed graphically as in text-fig. 5. It will be noted that the

form of the natural beat is essentially the same as that described for cilia whose movement has been artificially reduced to a much lower velocity (see Gray, 1928); at the same time we can now define the time relationships of the changes in form characteristic of the two strokes, and we can therefore determine with accuracy the effect of reagents on these processes. For the complete analysis of a single beat it is desirable to reach a much higher rate of exposure, but at present the use of a high-speed camera is impracticable, and many of the limitations which it would remove can also be avoided by methods to be described below. For recording the time interval between successive beats of comparatively low frequency the above technique is adequate (see text-fig. 7).

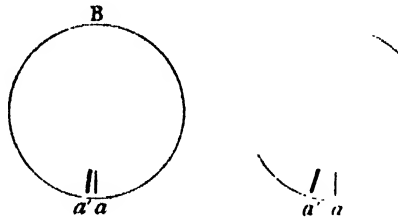
If a cilium is beating regularly at a given number of vibrations per second, it should obviously be possible to determine this frequency by illuminating the cilium for a very brief interval of time at a frequency exactly the same as that of the cilium's own motion. This is, of course, the principle of stroboscopic synchronisation; when the frequencies of the cilium and the illumination are identical the cilium will appear to be motionless. Further, by suitably adjusting the frequency of illumination a "slow motion" picture becomes available. Observations of this type have given valuable information, but before these results are described, it is advisable to mention certain features of stroboscopic analysis which are peculiar to ciliary movement.

In the first place, it is difficult, except in the case of the large abfrontal cilia of *Mytilus*, to isolate a single cilium in the field, and in the case of the *abfrontals* the frequency of vibration is too slow to allow of stroboscopic technique. The method is, however, well adapted for an analysis of the movements executed by individual cilia which naturally form part of well-defined metachronal field. In this case a given cilium reaches the zenith of its stroke a fraction of a second after the cilium in front of itself and a similar period of time before the cilium immediately behind it; in this way well-defined waves pass over the epithelium, the crests of the waves marking those cilia at the zenith of their stroke.

Before applying the stroboscopic method to the analysis of such a system it is useful to observe, under interrupted light, an inanimate system moving with the same angular velocity as the crest of a ciliated wave. Just as the musculature of the eye is responsible for the blurred image of a moving cilium observed under continuous illumination, so the properties of the retina and centres of optical sensation must be considered in attempting to analyse ciliary movement by stroboscopic methods.

If an object be brightly illuminated for a short period of time (e.g., 0.01 second), the sensation of an image on the retina is retained for a considerable period after the object has ceased to be illuminated. Persistent images of this type are due to the physical pro-

perties of the retina, but the extent to which the observer is conscious of such images is controlled by other factors. These phenomena are clearly illustrated during the observation of a ciliary field and it is for this reason that it is convenient to make a preliminary analysis of a comparable inanimate system. Starting with a circular white disc marked by a black line at its periphery (text-fig. 2A) and rotating it at about twenty times a second in a clockwise direction, the impression recorded under continuous illumination is a grey band at the periphery of the whole disc. If the disc is examined by intermittent light of a frequency of 20 per second, the grey band is replaced by a clearly defined and stationary

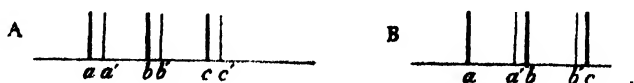


TEXT-FIG. 2.

image of the black line. If the frequency of the light is now very slightly decreased, the image of the line remains clear but appears to rotate slowly in a clockwise direction, and if the frequency of illumination is slightly increased beyond 20 per second, the image appears to rotate slowly in an anticlockwise direction. In both cases the observer is only conscious of one image of the line. Actually, in both cases, the eye registers more than one image but the sensation of duplicated images can only be obtained if the difference between the frequency of rotation of the disc and the frequency of illumination is sufficiently great. This can best be illustrated by reference to text-fig. 2. In text-fig. 2A the line on the disc has rotated from a position *a* to a position *a'* between two successive illuminations. At the moment of the second illumination, the eye records the primary image of the line at *a'* and is retaining a persistent image of the line at *a*. Of these images *a'* is the brighter since *a* is fading and it is the peculiar properties of the centres of optical sensation that all consciousness of *a* should be suppressed, for whenever a primary image of an object is close to a persistent image in this way, the observer automatically obtains the sensation of motion (from *a* to *a'*) and is not conscious of the persistent image. As the distance between a primary image and a persistent image increases so the powers of suppression are reduced and there comes a time when two or more images are clearly visible (text-fig. 2C), as in ordinary stroboscopic patterns. Patterns of this type are readily obtained when examining a ciliated field and it is necessary to avoid them by suitable adjustment of the stroboscope to such frequencies as enable the sensation of persistent images to be suppressed.

A second factor of technical importance lies in the fact that ciliary waves are moving with a very low absolute velocity. If the disc used in the above experiment be replaced by one which carries ten equally spaced black lines, and if the disc be rotated about once a second, we approach the actual time relationships of metachronal ciliary waves, since from 10 to 20 waves pass a given point per second. With such a disc it is possible to follow a given line for some distance round its circuit under continuous light, but it is impossible to focus the eye on a given spot on the periphery of the disc for any length of time; these phenomena are precisely those seen in the lateral epithelium on the gills of *Mytilus*. If the subdivided disc (rotating 10 times per second) be illuminated by intermittent light of

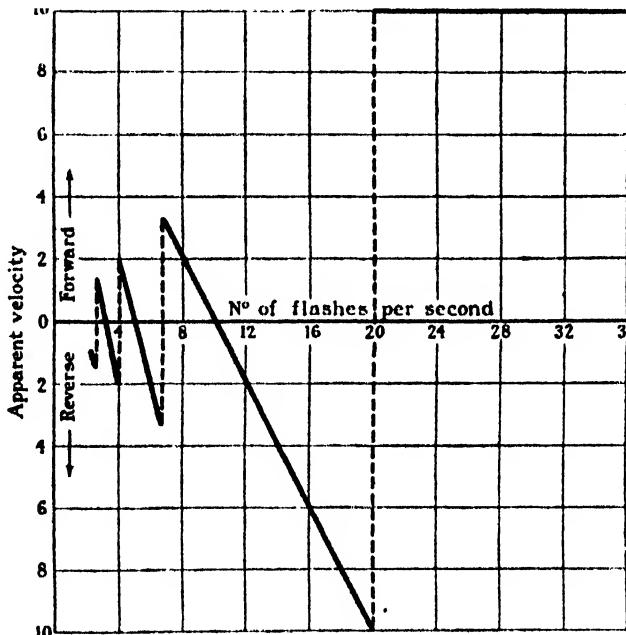
high frequency, the impression given is not materially different from that in continuous light. If the frequency of the illumination is allowed to fall to a value greater than but not far removed from 20 per second, the apparent direction and speed of movement of the individual lines remains unchanged and equal to the actual direction and velocity. As the frequency of illumination approaches 20 per second each successive image approximates to a position midway between two previous images. The actual sensation recorded by the observer at this point depends on the movements executed by the eye at the moment of observation. If the axis of the eye is stationary a well-defined stroboscopic pattern is seen, and a double number of images are visible, viz., 20. If the frequency of illumination is exactly 20 per second the stroboscopic pattern will appear stationary, but if the frequency be slightly greater or less than 20 per second the pattern appears to move anti-clockwise or clockwise respectively. Up to this point the observed phenomena are essentially the same as in the previous experiment, but if the axis of the eye is not stationary during observation, the field gives a different impression. During the observation of a moving object the tendency is for the axis of the eye to move in the direction of motion, and during intermittent illumination the observer naturally follows the bright primary images in their apparent direction of motion and remains unconscious of persistent retinal images. This fact is of considerable assistance in analysing ciliary movement since the absolute rate of movement involved is low. As long as the subdivided disc is not rotating too rapidly it is possible, under intermittent light, to follow the movements of the primary images clearly by moving the axis of the eye in the direction of movement, thus avoiding the sensation of stroboscopic patterns which arises if the axis of the eye remains stationary. This model of a rotating disc may conveniently be used to illustrate the essential method of observing a ciliated field. If the disc (with 10 radial lines) be rotated in a clockwise direction once a second, and be illuminated by a weak light at a frequency of 10 per second, a clearly defined and stationary image is seen of all the lines. As the frequency of the illumination falls, the lines remain bright but appear to rotate slowly in a clockwise direction and the observer is unconscious of any persistent images. Correspondingly, as the frequency of illumination is increased above 10 per second, so the primary images appear to move in an anti-clockwise direction—in each case the natural tendency is for the eye to follow a particular line in the apparent direction of motion. It will be noted that the apparent direction of motion depends on the relative position of pairs of successive primary images. Thus in text-fig. 3A, let  $a, b, c$  be the position of three primary images in respect to any fixed point, and let  $a', b', c'$  be the position of the lines at the next illumination; under these circumstances the mind associates image  $a'$  with  $a$ , and  $b'$  with  $b$  and gives the sensation



TEXT-FIG. 3.

of motion from left to right. In fig. 3B, however, the position of the second series of images is such that  $a'$  is nearer to  $b$  than to  $a$  and consequently the brain associates  $a'$  with  $b$  and  $b'$  with  $c$ , giving the impression of motion from right to left. If the interval between successive illuminations is such that  $a'$  falls immediately between  $a$  and  $b$ , three separate impressions can be recorded according to the movements executed by the eye. If the eye is kept stationary a stroboscopic pattern is recorded and the sensation of images

$a, a', b, b', c, c'$  is obtained simultaneously ; if, however, the axis of the eye is forced from left to right, the motion of a single series of primary images to the right can be recorded, or if the eye is forced from right to left the apparent direction of motion of the primary images is reversed. In actual practice the stroboscope is used at such frequencies as are slightly greater or less than the absolute frequency of the ciliary movements, and the phenomena of stroboscopic patterns can readily be eliminated. Until these principles are grasped, the examination of a ciliated field is liable to be confusing, whereas with a little experience extremely clear images can readily be obtained. A "slow motion" picture of the field can also be readily obtained when the frequency of illumination is slightly less than that of the frequency of beat of the cilia. The impression of a stationary field will theoretically be reached when the interval between two successive illuminations is equal to or is an exact multiple of one complete ciliary cycle (see text-fig. 4). It is convenient to start the illumination at high frequencies per second and to reduce this value gradually ; in this way it is possible to ensure that not more than one complete ciliary cycle has occurred between two successive illuminations. By such means the absolute frequency can be



TEXT-FIG. 4.—Diagram showing the apparent velocity and direction of movement of a series of metachronal waves, when illuminated by intermittent light of variable quantity. Ten waves pass a given point per second.

determined with considerable accuracy. Theoretically a still further reduction in the frequency of illumination initiates a cycle of events corresponding to that which begins when the frequency varies from twice the number of lines passing a point per second to a value which is equal to the latter (see text-fig. 4). In practice, however, a secondary factor intervenes. In order to obtain the sensation of continuous illumination free from undue flicker, it is necessary that the ratio of the frequency of illumination to the brightness of the field should not fall below a critical minimum. If this minimum is not maintained, the

observer is conscious of a series of discontinuous images and not of a permanently illuminated field. Consequently, if the frequency of illumination is falling during any given series of observations, it is necessary to reduce the intensity of the light in order to avoid flicker. Starting with a series of ciliated waves passing a given point at about 10 per second, we cannot usefully observe the field with frequencies of illumination much below about 6 or 7 per second—for at this point the limiting field is extremely dark—and by increasing its brightness we simply increase “flicker” to an excessive degree. The application of these principles to ciliary movement has shown that the most significant portion of text-fig. 4 consists between those frequencies of illumination which lie on each side of the point of true synchronisation. It will also be clear that for analysis by direct stroboscopic methods the higher the frequency of beat of the cilia the better, for this permits of a brightly illuminated field free from “flicker.”

The stroboscopic equipment at present being used consists of a stroboscopic disc fitted with a variable but balanced aperture mounted directly to the spindle of a small electric motor whose speed can be controlled by suitable rheostats. The lamp is mounted in a box below the motor and the light falls directly through the aperture of the disc on to the mirror of a microscope. The frequency of rotation of the disc is recorded directly by a speedometer. It will be noted that it is only possible to determine the frequency of ciliary beats by direct stroboscopic vision when their frequency exceeds about 6 per second. For speeds of this order or below it is necessary to use the shutter of the cinematograph camera as a stroboscope. The technique is costly and is only required on relatively few occasions. The principle involved is identical with that described. The camera carries a telescope whereby the images falling on the film can be seen during all the exposures, and the speed of the camera is controlled by a calibrated rheostat giving at will from three to eight pictures per second. The camera is started at its normal speed 16 per second, the resistance is then brought into the circuit and is very slowly increased until the frequency has fallen to the minimum value of 3 per second; the speed is then very slowly increased. After development, the film can be printed and, at convenient spots on the positive, the actual speed of the original exposures is marked by reference to the time markings on the negative. On projecting the positive, the film is stopped at the point of minimum movement and by reference to the timing signals the actual frequency of vibration is known.

It will be obvious that considerable caution is required in interpreting the true direction of motion and the actual velocity of movement of metachronal waves from projected films whose original frequency of exposure is not accurately known with reference to the true frequency of the waves per second; it must be remembered that the shutter of the camera is acting as a stroboscope,

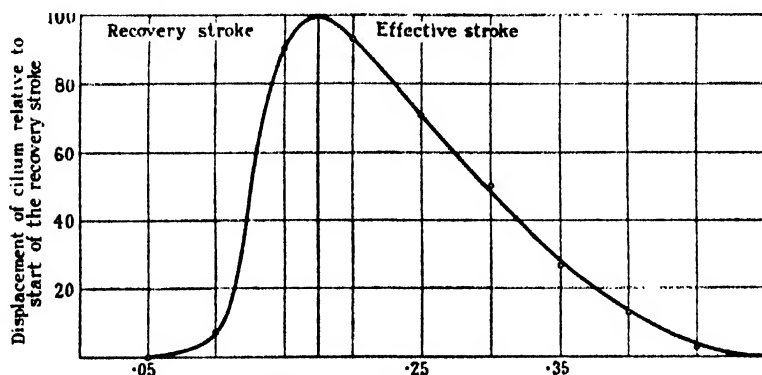
and the sensation given by the projected photographs will give the stroboscopic sequence of events and not necessarily the true sequence.

## II. *The Nature of the Ciliary Beat.*

The difference in the form of a cilium during the two phases of its beat has been described elsewhere (Gray, 1922, 1928). Apparently all those cilia which are capable of doing work against the resistance of an aqueous medium move through their effective stroke as rigid rods and through their recovery strokes as though they were readily flexible. How far this difference is due to an active change in the elastic properties of the cilium and how far it is due to permanent structural organisation is unknown. By means of a timed cinematograph film it has been found possible to take twelve photographs during a single beat of the large abfrontal cilia on the gills of *Mytilus*. By removing a single gill filament and laying it on its side it is not difficult to view a single cilium in profile. It will be noted (Plate 20) that in many essential features these photographs confirm the results previously obtained by indirect methods of observation. The recovery stroke starts by a slow bending of the base of the cilium (Plate 20, photographs 1 and 2) which is propagated along the cilium at an increasing speed (Plate 20, photographs 4 and 5) until the cilium is rigidly extended at right angles to the surface of the tissue. In photograph 5, a slight curvature can still be detected at the tip of the cilium. On very few occasions has it proved possible to photograph a cilium between the positions shown in photographs 4 and 5, from which it may be inferred that the time interval between these two positions is extremely short. The recovery phase of the ciliary beat therefore starts relatively slowly and ends slowly—but between these two periods is one of rapid acceleration during which the contraction wave passes along the cilium at a relatively rapid rate. The absolute rate in the propagation of the contraction wave is, however, very low. The length of an average abfrontal cilium is of the order of  $100\ \mu$  and the recovery stroke occupies approximately 0.2 second, hence the *average* rate at which the bending wave passes along the cilium is 0.5 mm. per second, a figure much lower than in any known muscle fibre. The effective stroke of an abfrontal cilium is illustrated by Plate 20, photographs 6 to 10; during its whole course the cilium is seen to be rigidly extended; during this phase the angular velocity of the cilium gradually increases and finally declines towards the end of the stroke. It will be noted in photograph 5 that the base of the cilium may start to move forward before the rest of the cilium is fully extended. This is not the case in other types of cilia. The whole beat (both recovery and effective strokes)



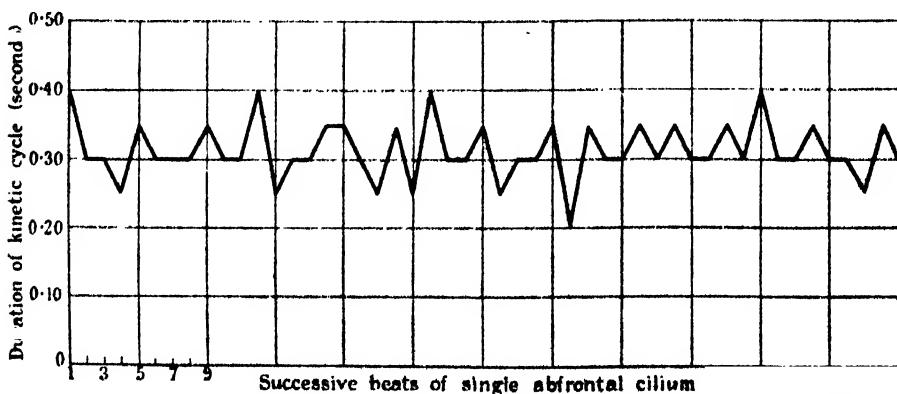
can be graphically represented as in text-fig. 5 where the ordinate represents the position of maximum curvature for the recovery stroke, and the angle which



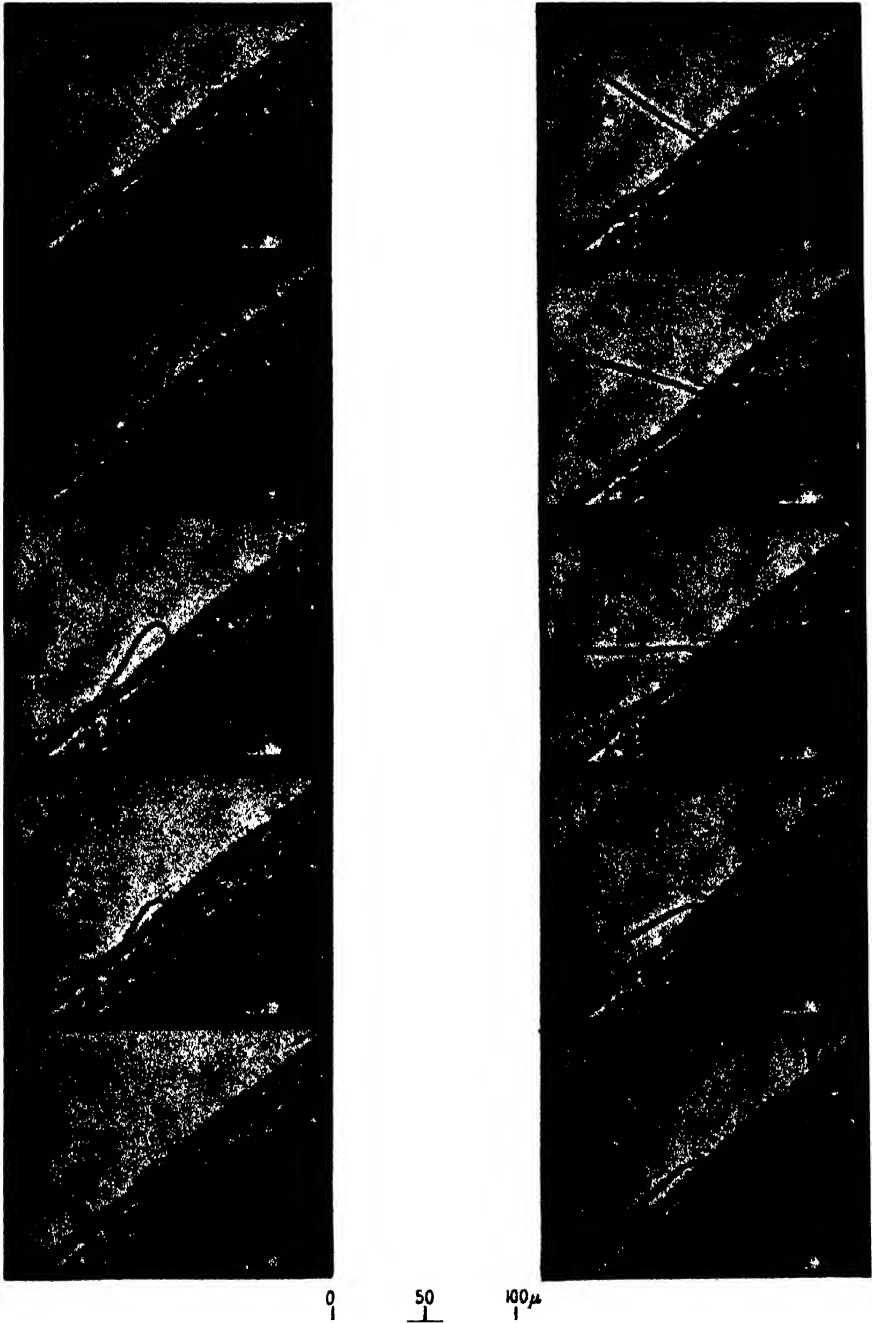
TEXT-FIG. 5.—Record of a complete beat of an abfrontal cilium. The recovery stroke is obtained by plotting the position of maximum curvature at different times. The effective stroke is recorded by plotting the angular displacement of the cilium relative to the position of rest; the total displacements of both phases being regarded as 100.

the cilium makes with the base line for the effective stroke; in both cases the abscissa represents time. In one respect the records are not typical of cilia in general; in most cases the duration of the effective stroke is considerably less than that of the recovery stroke, but in the case of the abfrontal cilia here considered the reverse is the case.

The period of time occupied by successive beats is remarkably constant. Text-fig. 6 shows, diagrammatically, the duration of the kinetic cycle of each of



TEXT-FIG. 6.—Showing fifty successive beats of an abfrontal cilium. Each beat was succeeded by another without any measurable intervening pause.



Ten successive photographs of a single beat of an abfrontal cilium of *Mytilus*. Photographs 1-5 show the form of the recovery stroke; photographs 6-10 show the form of the effective stroke. Interval between each photograph 0.05 seconds.

(Facing p. 322.)



50 successive beats. When grouped in successive periods of five beats the times (accurate to 0.05 second) occupied, in this particular case, are as follows :—

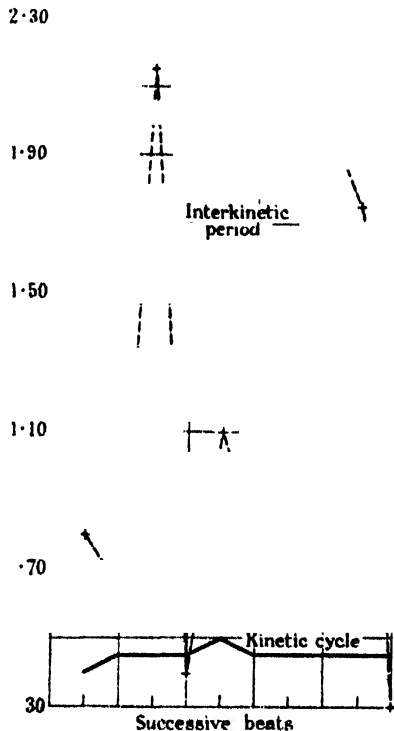
1.60, 1.55, 1.55, 1.60, 1.60, 1.40, 1.60, 1.60, 1.65, 1.65 seconds.

As in many other similar observations, one kinetic cycle follows another without any measurable period of rest, so that the frequency of beat of an individual cilium remains fairly constant over a considerable period of time. In other cases, however, abfrontal cilia have a very irregular frequency, but it is evident that the irregularity of the rhythm is due to an irregularity in the period of time which elapses between each kinetic cycle and not in the period occupied by the phase of mechanical movement. An example of this type is illustrated in Table I and by text-fig. 7.

In no case has an abfrontal cilium exhibited simultaneously a well-defined interkinetic period and a constant frequency of beat per second. Constancy of rhythm is, however, maintained when the interkinetic periods are abolished. How far these latter periods can be controlled by experimental means remains to be investigated.

The abfrontal cilia of *Mytilus* are of interest, because they are one of the few instances in which it is possible to record the form and behaviour of an individual cilium which is beating at its normal rate and under normal conditions; in nearly every other case, cilia are too small or too crowded together to permit of individual observation. It is well known, however, that a sheet of ciliated

epithelium not infrequently exhibits a periodicity of movement, although it is impossible to see the individual cilia which are responsible. An epithelium of this type is present as the *lateral* ciliated epithelium on the gills of *Mytilus* and it is interesting to note that the data derived from the study of the



TEXT-FIG. 7.—Ten successive beats of an abfrontal cilium showing well-defined but very irregular pauses between each beat. Note the relative constancy of the successive kinetic cycles.

Table I.

	Successive beats.									
Duration of kinetic cycle, in seconds .....	0.40	0.45	0.45	0.45	0.50	0.45	0.45	0.45	0.45	0.45
Duration of interkinetic cycle, in seconds .....	0.80	0.65	2.15	0.40	1.10	0.75	1.15	2.00	1.75	0.30

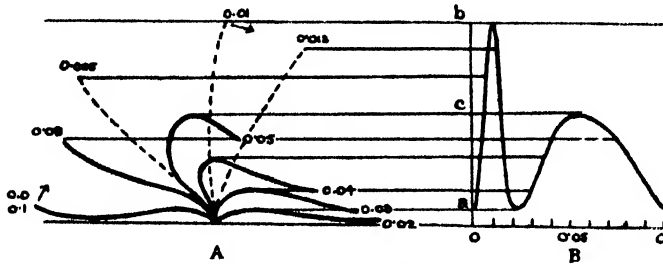
individual *abfrontal* cilia throw considerable light on the behaviour of *lateral* epithelium.

### III. *Metachronal Rhythm.*

A cursory examination of a healthy gill of *Mytilus* reveals that the surface of the *lateral* ciliated epithelium exhibits a series of regular waves (Plate 21, figs. 2A-C) which pass along the filament from one end to the other. These waves are composed of actively moving cilia whose plane of beat is at right angles to the lateral surface of the filament of the gill, and (as illustrated by the photographs in Plates 21 and 22, figs. 2 and 3) at right angles to the page in the plane of vision. If these waves are focussed carefully under a  $\frac{1}{4}$ -inch objective, they can be seen to consist of two parts; the front of the wave is marked by a sharp spike behind which is a rounded crest (Plate 22, fig. 3). The dual nature of individual waves is also clearly seen towards the centre of fig. 2B, Plate 21. If, instead of viewing the lateral epithelium in profile, the gill filament be laid on its side, with the lateral epithelium towards the observer, the waves appear as a series of moving lines or as a moving spiral according to the exact focus. In Plate 22, fig. 4, the lines are clearly visible. If viewed in this way under a current of air containing CO<sub>2</sub>, the activity of the epithelium rapidly falls and eventually all trace of the waves and of rhythmic activity is lost. In this condition a few isolated cilia can usually be observed in motion after the rest have ceased to beat. Such cilia show clearly that the amplitude of beat is approximately 180° and not 90° as in the case of the *abfrontal* cilia already described; it is also clear that the effective stroke is more rapid than the recovery stroke. It starts with the tip of a cilium at a point P (Plate 22, fig. 4) and ends at E. It is very difficult to assess the number of cilia which participate in the formation of one complete wave, but it is probably not less than 20 or more than 50.

The regularity of a metachronal wave indicates that each constituent cilium is not beating independently of its neighbours but is always a definite phase interval behind the cilium on one side of it and ahead of the cilium on the other

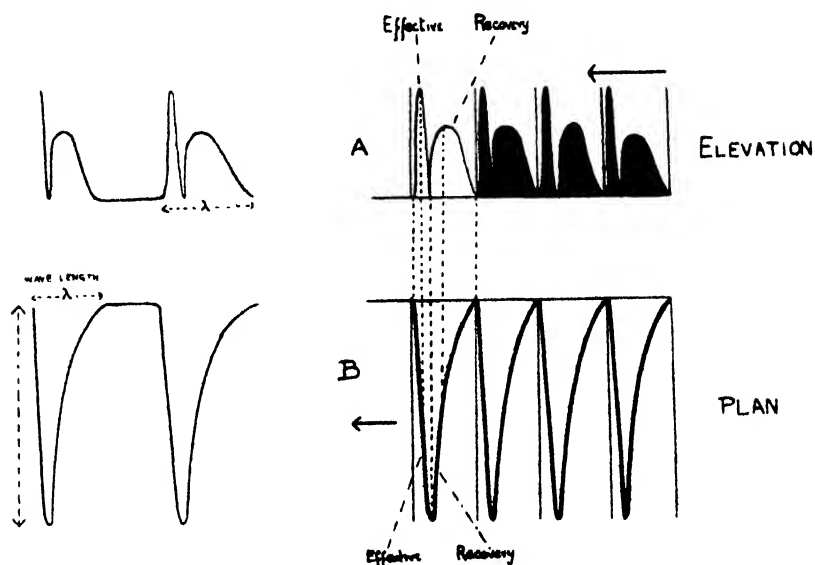
side. We may assume, for the moment, that this difference represents a fixed period of time. If this be so, then it is simple to reconstruct the form of the observed metachronal waves from the data derived from a study of the *abfrontal* cilia. Text-fig. 8, A, represents a profile view of a cilium which differs



TEXT-FIG. 8.—Showing the relationship between the form of beat of a cilium (A), with the form of the resultant metachronal wave (B). The cilium is viewed in a plane at right angles to the plane of movement, the cilium beating in the plane of the paper. The metachronal wave is viewed in profile, its individual cilia beating in a plane at right angles to the plane of the paper. The wave is composed of cilia each of which differs in phase by a constant time difference from its neighbours. In A the phases of the effective stroke are dotted, the phases of the recovery stroke are drawn in full lines; the numbers denote the times after the beginning of the effective stroke at which the phases are reached.

from an *abfrontal* cilium in that its amplitude is  $180^\circ$  and its effective stroke is approximately four times as rapid as its recovery stroke; in other respects the beat is exactly comparable to that illustrated in Plate 20. If such a cilium were viewed in the plane of its beat the apparent tip of the cilium would execute a series of harmonic movements between *a* and *b* (text-fig. 8, B), and between *a* and *c*, alternately. During the effective stroke the tip would appear to move rapidly from *a* to *b* and back again to *a*; during the recovery stroke it would appear to move slowly from *a* to *c* and back again. A series of such cilia in line and each being a constant small period of time behind each other in phase, would exhibit an outline such as is shown in text-fig. 8, B, the high crest being cilia in various phases of their effective stroke, the lower rounded crest being cilia in various phases of the recovery stroke. If we start with a series of stationary cilia and each one starts its beat (in sequence) a definite period of time before the next in line, a double-crested wave will be seen to pass over the series when these are viewed in profile. There can be little doubt that the form of the metachronal wave is the direct consequence of the form of beat of the individual cilia. It is of interest to note that the rate of propagation of the wave of curvature which passes along a lateral cilium during its recovery

stroke is of the same order of magnitude as that of the much larger abfrontal cilia, viz., 0.5 mm. per second.



TEXT-FIG. 9.—Diagram showing the form of metachronal waves when viewed (A) tangentially to the epithelium in the plane of the ciliary beat, (B) at right angles to the epithelial surface.

If a series of metachronal waves is to pass over an epithelium it follows that the frequency at which the waves pass a given point per second must be the same as the frequency of beat of the individual cilia and the frequency of beat of all the cilia concerned must be the same. It has already been shown that the duration of the kinetic cycle of an abfrontal cilium, under standard conditions, is relatively constant, whereas interkinetic periods, if present, are of variable duration. The same phenomena are encountered in the lateral cilia. When a small area of lateral epithelium is examined under intermittent light, it is possible so to synchronise the illumination with the frequency of beat as to obtain the impression of a stationary wave. Thus at 15° C. the frequency is found to be of the order 15 to 20 vibrations per second and for any given spot the frequency remains the same for appreciable periods of time.

Experiment number.	Frequency of beat of lateral cilia per second.			
1	17.7	18.3	16.7	17.7
2	16.7	16.0	15.0	16.1
3	15.7	16.3	15.0	16.0



FIG. 1. Microcinematograph with timing mechanism.

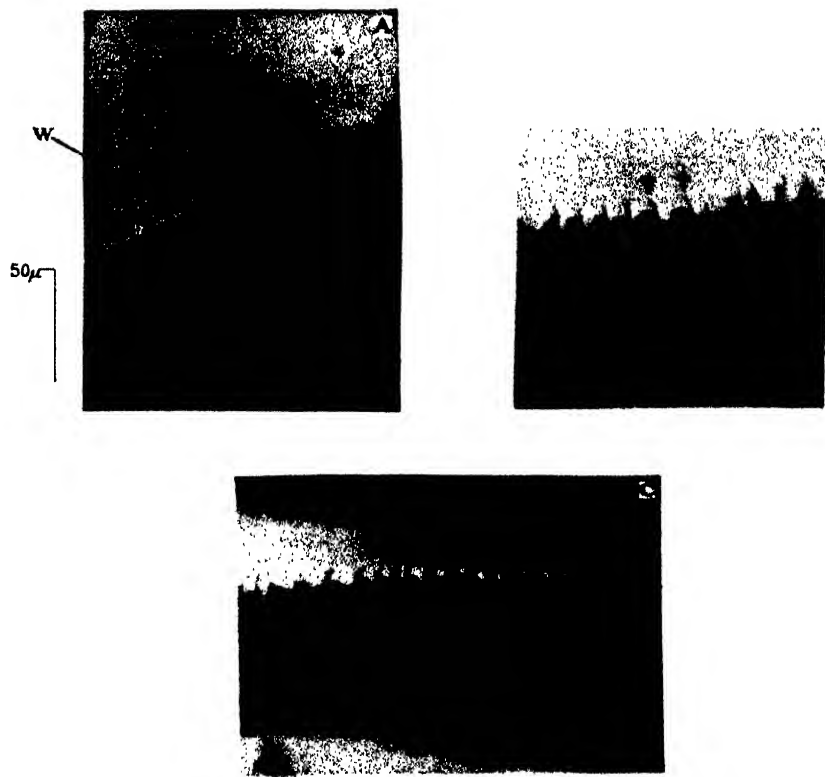


FIG. 2.—Profile view of metachronal waves. In A the waves are fairly regular in wave-length and there is no interkinetic pause. In B a well-marked interkinetic pause is seen: note the form of the individual waves ( $\dagger$ ). In C the lack of rhythm can be seen in at least two places ( $+$ ).

(Facing p. 326.)



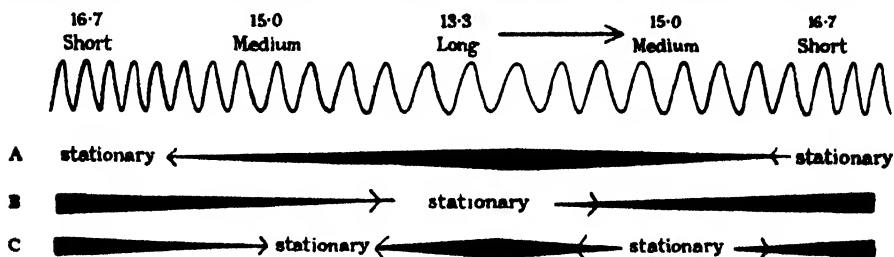


One metachronal wave normally follows another without any intervening period of ciliary rest (Plate 21, fig. 2A). Occasionally, interkinetic periods can be detected (Plate 21, fig. 2B) where one wave is separated from the next by a definite interval; as might be expected from the behaviour of the abfrontal cilia, waves of this nature tend to be irregular in frequency.

The wave-length of metachronal waves varies somewhat but is of the order of  $10\ \mu$ , so that if 16 waves pass a given point per second, the rate of propagation of the wave is  $160\ \mu$  per second. In comparison to the rate of propagation of a nervous impulse or the spread of contraction over a muscle fibre, this velocity is extremely low. It may be remembered, however, that whatever be the nature of ciliary transmission it has to pass from cell to cell in the epithelium and as each cell is approximately  $10\ \mu$  in width, the impulse must pass from cell to cell in 0.06 second, which is not so far removed from that observed in nervous elements or from a nerve to a muscle fibre. Against this explanation of the slow rate of ciliary transmission is the fact that there is no apparent change in the form of a metachronal wave as it passes over a cell boundary, and that transmission over the surface of a protozoon is of the same order of velocity as is observed in a multicellular epithelium. It is extremely doubtful whether a network of excitatory nerve fibres such as is postulated by Grave and Schmidt (1925) can possibly be concerned with metachronal rhythm (see Bhatia, 1926).

The facts so far described are based on the behaviour of individual cilia or of small lengths of lateral epithelium of the order of  $50\ \mu$  in length. If a comparatively long strip of epithelium be examined by intermittent light it is found that the wave-lengths and frequency of the metachronal waves is not absolutely uniform over the whole surface. Two types of variation have been observed. In two cases it was quite clear that the wave-length ( $\lambda$ ) of the waves varied without any alteration in the frequency; under these circumstances each wave on reaching a particular position in the epithelium changed its shape relatively abruptly and thereby increased the wave-length which was again reduced after passing over the particular region of the epithelium concerned. The simplest explanation appears to be that regions of long wave-length were characterised by a reduction in the time which elapsed between successive cilia beginning their effective stroke. This phenomenon is distinctly uncommon in comparison to the almost universal type of metachronal variation now to be described. When successive regions of a strip of lateral epithelium are examined under intermittent light the frequency of ciliary beat and therefore of metachronal waves is found to vary from place to place

along the epithelium, and simultaneously a variation in wave-length can be observed. High frequency is correlated with short wave-length. As a rule there is no sudden change from short frequent waves to those of lower frequency and longer length, but as shown diagrammatically in text-fig. 10, and photographically in Plate 22, fig. 5, the change is gradual. It is not easy to measure the



TEXT-FIG. 10.—Illustrating a typical case of variation in the frequency and wave-length of metachronal waves on the lateral epithelium of *Mytilus*. At the extreme right and left are regions of short wave-length and high frequency (16.7 per second); in the centre are long waves of lower frequency (13.3 per second); between the short and long waves are waves of intermediate length and frequency (15.0 per second). The actual direction of the moving waves is from left to right. When viewed by intermittent light of frequency 16.7 per second (A) the short waves appear motionless while the apparent direction and velocity of the remainder is indicated by the direction and thickness of the first arrow. When viewed by light of 13.3 per second (B), the short waves appear to advance with a velocity which increased with diminution of wave-length. When illuminated with a frequency of 15.0 a second (C), waves appear to converge with diminishing velocity towards the area of stationary waves, or to diverge with increasing velocity according to the orientation of the longer and shorter waves in respect to those of intermediate length. This figure is a composite record of actual observations.

wave-length except in photographic records, and as a rule the frequency of waves was determined stroboscopically and not by means of a camera. The variation in wave-length can, however, be seen qualitatively by synchronising the intermittent light with a selected region of the epithelium. If, for example, a group of short waves are made to appear stationary (as on the right of text-fig. 10, A, or Plate 22, fig. 5) the waves to the left of this region can be seen to move with increasing velocity towards the longer waves of lower frequency (shown in the centre of text-fig. 10), and as they move, so the wave-length gradually increases. The phenomenon is curiously well defined and visible in a projected cinema film whose frequency of exposure has been suitably adjusted. The facts show clearly that the actual rhythm of a metachronal field is by no means as regular as might be supposed, since the number of ciliary beats and metachronal waves passing different points per second varies. However gradual may be the

transition from a region of one frequency to a region of higher or lower frequency, it seems certain that periodic disturbances in rhythm and in the form of the successive waves must occur. Except in extreme cases, such disturbances are not readily detected by visual observation under intermittent light; they are, however, visible on individual frames of a cinematograph record (see Plate 21, fig. 2c).

Although the wave-length of a metachronal wave passing a given area in an epithelium is associated with the relative frequency of beat of the constituent cilia, this correlation is a fixed property of the tissue and is independent of the absolute frequency of the cilia. For example, if the temperature of the tissue be raised from 15° C. to 25° C. no detectable change occurs in the wave-length although the frequency of beat rises materially. At 5° C. the frequency is too low to be measured accurately by means of the stroboscope, and must be of the order of not more than seven or eight a second; at 25° C. the frequency is at least 20 per second, and yet no change occurs in the wave-length of the metachronal waves. The independence between wave-length and absolute frequency of the cilia is in curious contrast to the transmission of waves along a single flagellum or along the tail of a spermatozoon (see Gray, 1928).

It is not easy to weld these facts into a satisfactory theory of ciliary transmission. If each cilium be regarded as stationary except when excited by an external stimulus, and if a series of such external stimuli are passing along the epithelium from one end to the other, metachronal waves would result whose frequency would be the same as the frequency of the stimuli and whose wave-length would vary with the speed at which the stimulus travelled along the epithelium. Against this view there are three objections: (i) Metachronal waves can arise or be suppressed at any point along an epithelium, and cannot be traced to a centre from which the exciting stimulus can be shown to arise; (ii) isolated ciliated cells are almost invariably active, and when *in situ* in an epithelium the only form of nervous control to which they are at all frequently susceptible is of an inhibitory nature; (iii) the rate of metachronal transmission is very much lower than that of any known nervous impulse. If metachronal rhythm is not the result of an extrinsic series of propagated stimuli, it must presumably be due to the activity of the cilia themselves. For example, if a cilium is automatically excited when its neighbour has reached a definite stage in its own kinetic cycle, the metachronal waves could be regarded as having an intrinsic origin in the actual ciliary cycle. Starting from a stationary series of cilia and exciting one, the others will proceed to beat in metachronal rhythm. If the frequencies ( $f$ ) of all the cilia are equal and if one complete

beat occupies  $T$  seconds, and the phase difference at the moment of response of one cilium to a neighbouring cilium be  $t$  seconds, then  $T/t$  cilia will be in a state of motion during the period of one complete ciliary cycle. If each cilium is  $k\mu$  distant from its nearest neighbours the wave-length of the metachronal waves will be  $k/ft$  microns. If the number of cilia composing a typical metachronal wave lies between 20 and 50, the phase interval between one cilium and its immediate neighbours must lie between 0.001 and 0.003 second. Alternatively, it is conceivable that the stimulus which arises from a moving cilium is due to its mechanical drag on its stationary neighbour and that this will be effective when there is a critical phase difference in position between the two. In this case the wave-length of the metachronal waves will be  $kd_1/fd$  where  $d_1$  is the velocity of the cilium in  $\mu$  per second, and  $d$  is the distance a cilium moves before mechanically exciting its neighbour. In both cases the wave-length is inversely proportional to the frequency if other factors are constant. The reciprocals of  $t$  and  $d$  can be looked upon as a measure of the excitability of the cilia, so that the wave-length may be regarded as directly proportional to this factor. It has been shown that when the temperature of the tissue changes, there is no apparent change in the wave-length of the metachronal waves; according to the hypothesis here put forward it may be concluded that the expression  $ft$  or  $fd$  remains constant, and this means that  $f$  and  $1/t$  have been affected to exactly the same extent. This suggests that both the frequency of beat and the excitability of the tissue form part of one indivisible mechanism.

It will be recalled that the velocity of transmission of the waves is of the same order of magnitude as the velocity of a moving cilium. It is not difficult to imagine how one cilium may affect its neighbours. If a cilium is beating slightly faster than its neighbour there will occur periods at which they would, if completely independent, be completely out of phase with each other; with such fine structures, closely applied to each other, each will, however, exert a viscous drag on the other which will operate in such a way as to keep the phase difference between them below a certain critical limit—the faster moving cilium will constantly tend to accelerate its slower neighbour and the slow cilium to slow up the more rapid cilium. Under such conditions metachronal rhythm might be maintained by the principle of minimum interference although periodic loss of rhythm would occur. It is, however, not easy to explain why the direction of metachronal waves is irretrievably fixed for any given epithelium and depends solely on the structural orientation of the tissue. If the waves were solely of a mechanical origin, they could travel equally well in either

direction unless there were a structural difference between the two sides of the cilium. Even then it would be difficult to explain the behaviour of those tissues, not found in *Mytilus*, where the waves travel in a line which is not at right angles to the plane of beat of the cilia.

*Summary.*

1. A technique is described for obtaining a photographic record of a single living cilium and of recording the position of the cilium at suitable intervals of time.

2. Cilia which are vibrating 10 or more times per second can be examined stroboscopically if suitable means are adopted for eliminating persistent retinal images.

3. Under standard conditions, the kinetic cycle of all the cilia examined was constant for a given cilium over a considerable period of time. At 15° C. the abfrontal cilia of *Mytilus* have a cycle of approximately 0.35 to 0.45 seconds; the lateral cilia 0.1 to 0.05 seconds.

4. The form of the effective and recovery strokes of normally beating cilia are essentially the same as those observed in cilia whose rate of beat has been artificially reduced to a much lower level.

5. The wave of curvature which passes along an abfrontal or lateral cilium during the recovery stroke travels with an average velocity of approximately 0.5 mm. per second.

6. When successive ciliary beats are separated by interkinetic pauses, the latter may be of very variable length although the periods of kinetic activity remain constant.

7. The form of a metachronal wave of cilia can be reconstructed from the form of beat of its constituent cilia. Metachronal waves on the lateral epithelium of *Mytilus* travel with an approximate velocity of 160  $\mu$  per second at 15° C., and with a frequency of 16 per second.

8. The wave-length and frequency of metachronal waves varies at different parts of the epithelium. Short wave-length is correlated with high frequency, but the correlation is not absolute, for when the frequency is increased by raising the temperature the wave-length remains unchanged.

9. The difference in phase between two adjacent cilia in the lateral epithelium lies between 0.001 and 0.003 seconds.

10. The ability of an epithelium to transmit metachronal waves probably lies in a mechanism which is part of the kinetic apparatus of each cell and not in any extrinsic system of neural control.

The expenses of this investigation were in part defrayed by Columbia University, New York City, and partly by a grant from the Government Research Fund. To both these sources I would like to express my thanks.

## REFERENCES.

- Bhatia, D. (1926). 'Quart. J. Micro. Sci.,' vol. 70, p. 681.  
 Grave, C., and Schmidt, F. O. (1925). 'J. Morph.,' vol. 40, p. 479.  
 Gray, J. (1922). 'Proc. Roy. Soc.,' B, vol. 93, p. 104.  
 Gray, J. (1928). "Ciliary Movement," Cambridge.

612.741.15:611.864.I.

*The Contraction of the Extrinsic Muscles of the Eye by Choline and Nicotine.*

By W. S. DUKE-ELDER and P. M. DUKE-ELDER (from the Department of Physiology and Biochemistry, University College, London), for the Physiology of Vision Committee, Medical Research Council.

(Communicated by C. Lovatt Evans, F.R.S.—Received September 24, 1930.)

It has long been known that choline, acetylcholine (Reisser, 1921), and nicotine (Langley, 1906-14) contract the normal striped muscle of *Sauropsidae* (frog, fowl, etc.), and it has been recently demonstrated that a similar reaction occurs in the foetal muscle of mammals (Rückert, 1930), but hitherto it has never been shown to occur in the muscles of the fully-developed mammalian. It has also been well established that the voluntary muscles of mammals, after degeneration of the motor nerves, exhibit a "pseudo-motor" contraction on the injection of choline (Frank, Northmann and Hirsch-Kauffmann, 1922-23), a contraction which appears analogous in origin and in nature to that first described in the tongue by Vulpian and Phillippeaux (1863), and in the muscles of the limbs by Sherrington (1894), which occurs on stimulation of the sensory roots after the motor roots have degenerated. Recent writers in discussing the theoretical basis of these phenomena have stressed the point that this type of contraction occurs in mammals only after degeneration of the motor nerves, and have based some of their conclusions upon this assumption. The following experiments show, however, that this statement is not universally true, and that the extrinsic muscles of the eye form an exception to the general rule.

The matter arose as a side-issue during an extended research on the mechanism controlling the intra-ocular pressure, when anomalous changes were noted while investigating the effect of choline and acetylcholine upon the pressure in the eye. These experiments are recorded in a separate publication (Duke-Elder, 1930): it is sufficient for the present purpose to say that in experiments upon anæsthetised dogs, while small doses of choline such as produce a depressor effect when injected intra-venously (0.2 c.c. of a 1 in 20 solution) give rise to a fall in the intra-ocular pressure of the order which would be expected from the events in the vascular circulation, larger doses, on the other hand, lead to an increase in the intra-ocular pressure much larger than could be explained by any vascular events. In order, therefore, to reduce the number of variables with which we were dealing, the technique was extended to the perfusion of the eye with an artificial circulation, whereby the conditions in the general

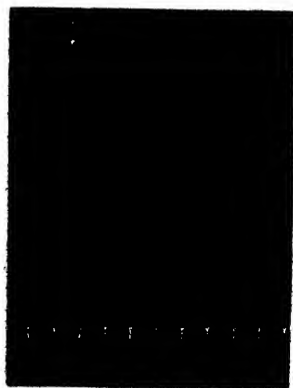


FIG. 1.—The effect of choline (0.1 c.c., 1 in 20) on the intra-ocular pressure in the artificially perfused dog's head. Upper curve, blood pressure; lower curve, intra-ocular pressure.

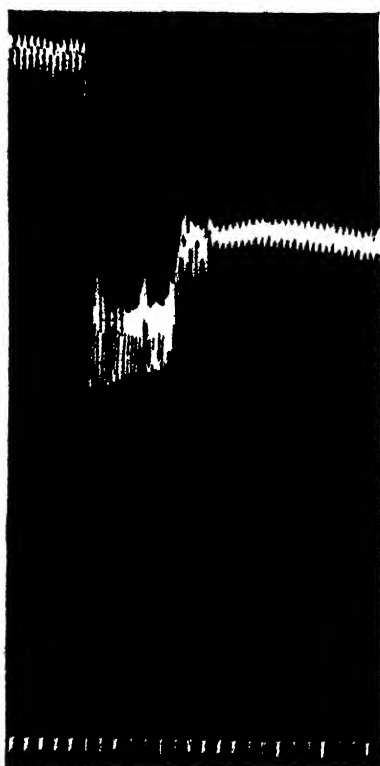


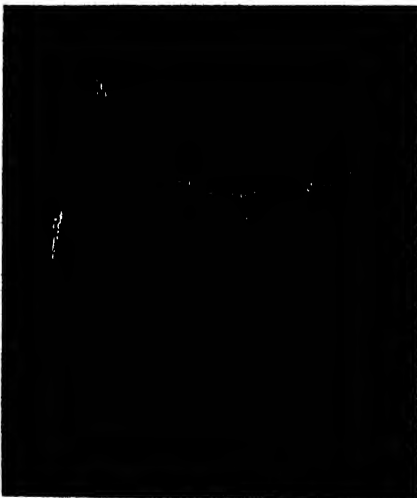
FIG. 2.—Contraction of a rectus muscle with acetylcholine (0.1 c.c., 1 in 10,000). Upper curve, blood pressure; lower curve, muscle record.



circulation were kept constant (Duke-Elder, 1930) ; and fig. 1 shows that in these circumstances, even when we would have expected a fall in the intra-ocular pressure owing to a local vasoconstriction when the pressor component of choline was elicited, a rise was obtained. The most significant feature was that this rise was accompanied by a movement of the base-line in the tracing registered by the optical manometer denoting a movement of the eye in the direction of enophthalmos.

Such a movement suggested that the pressure effects caused by any vascular reaction were being masked by some predominating influence which seemed necessarily to be that of muscular activity. A movement of the eye in this direction could be caused by a contraction of the recti muscles and the retractor bulbi, or by a relaxation in the tone of the plain muscle of Müller, but since the reaction was accompanied by a pronounced rise in the intra-ocular pressure it seemed evident that the first of these groups of muscles was implicated.

In a further series of experiments one or other of the recti muscles was therefore detached from its anterior attachment into the sclera, and connected with a recording lever. Fig. 2 shows the short, sharp contraction obtained on the injection of acetylcholine, and fig. 3 the much more prolonged effect obtained



A. injection (intra-venous) of 0.2 c.c. of 1 in 20 solution choline chloride.



B, repetition of injection of choline as at A, 3 mins. after an intra-venous injection of 0.1 c.c. of 1 in 1,000 adrenaline.

FIG. 3.—The effect of choline (0.1 c.c., 1 in 20) on the rectus muscle before and after adrenaline in the dog.

with choline. It is interesting that in quite a considerable percentage of the experiments, after the initial contraction has passed its height, the muscle went into a prolonged slow rhythmic contraction of the type seen in fig. 4. The previous injection of adrenaline caused a slight increase in the degree of

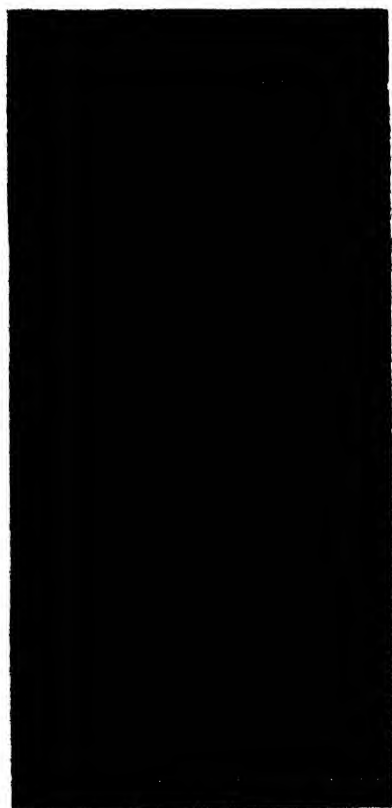


FIG. 4.—Rhythmic contractions after the initial contraction with choline (0.25 c.c., 1 in 20). Rectus of dog.

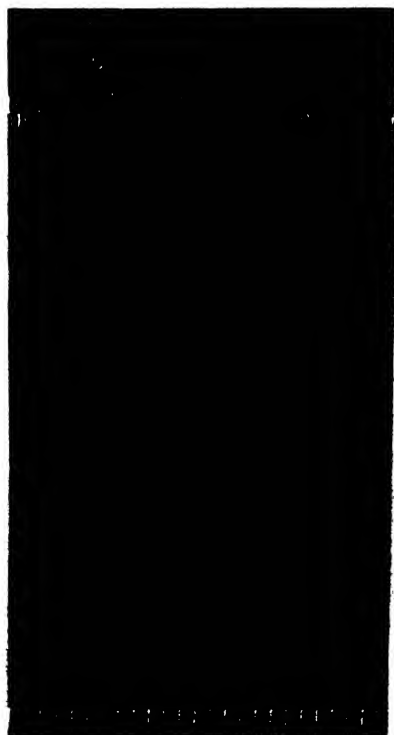


FIG. 5.—The contraction of the rectus with 0.1 c.c., 1 in 20, choline after 0.5 c.c., 1 per cent., atropine (dog).

the contraction (fig. 3). In further experiments atropine was injected intra-venously, which abolishes the depressor (muscarine-like) component of choline, and fig. 5 shows that on the subsequent injection of choline the muscular contraction took place as before. Nicotine was then injected intra-venously and it was found that the subsequent action of choline in contracting the recti was abolished. As a last observation curare was given, and fig. 6 shows that a subsequent injection of choline evoked no response. Experiments showed that similar reactions are obtained in the cat.

It was obviously of interest to compare these results with the variations in the intro-ocular pressure recorded after the administration of nicotine. The action of this drug in (initially) stimulating sympathetic activity is seen in the eye in a pronounced vaso-pressor effect and in a contraction the plain muscle of the orbit, the latter of which causes an exophthalmos. Fig. 7 illustrates the rise in the general blood pressure which follows its injection, accompanied

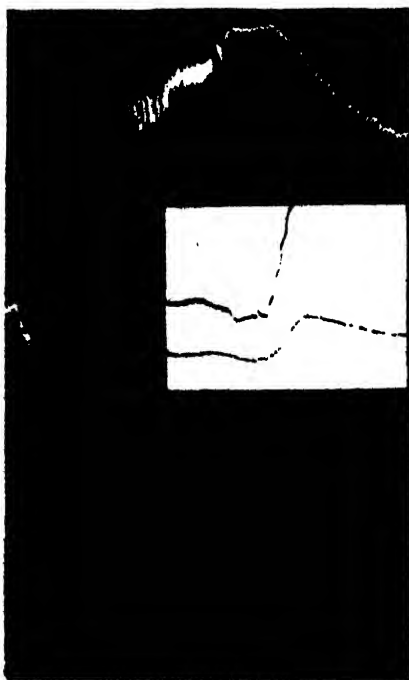


FIG. 7.—The effect of 0.2 c.c., 1 per cent., nicotine on the intact dog.

FIG. 6.—The effect of 0.1 c.c., 1 in 20, choline upon the rectus muscle after 2 c.c., 2 per cent., curare (dog). Upper curve, blood pressure; lower curve, muscle record.

by a rise in the intra-ocular pressure so steep that the tracing leaves the film. This abrupt rise in the pressure of the eye would seem obviously due partly to the effect of the rise in the general blood pressure, and partly to the contraction of the plain muscle round the globe, the action of which is seen in the movement of the base line in the sense opposite to that occurring with choline (see fig. 1). A tracing of a rectus muscle, however, shows clearly that a slow, tonic contraction of the voluntary muscle also occurs. In fig. 7, which represents the effect of a small dose of nicotine injected intra-venously (0.5 c.c. of a 1.0 per cent. solution), the long, slow contraction of the rectus is interrupted

by an abrupt relaxation near the commencement of the reaction ; this interruption we interpret as a carrying forward of the muscle by means of its fascial connections with the eyeball in the act of exophthalmos. With larger doses of nicotine, on the other hand, when a dissection is made freely separating the eye from its attachment to the globe, a definite and strong contraction of the rectus is recorded resembling in every respect that obtained with choline both in the cat and in the dog (fig. 8). It would appear, therefore, that relatively

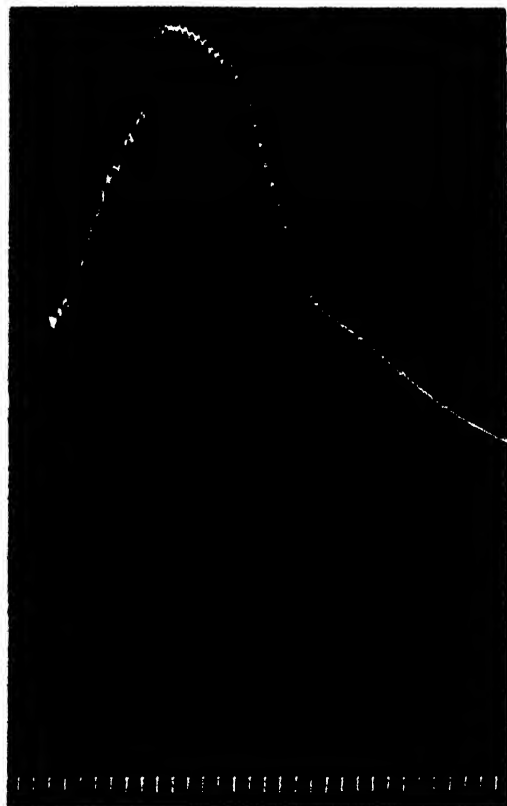


FIG. 8.—Contraction of the rectus with 1·0 c.c., 1 per cent., nicotine (cat). Upper curve, blood pressure ; lower curve, intra-ocular pressure.

large doses of nicotine are necessary to produce a pronounced contraction of the recti ; and that the contraction which is elicited with smaller doses is masked by the opposing activity of the plain muscle supplied by the sympathetic.

*Contraction of the Muscles in Vitro.*—A series of experiments were then undertaken to study the effect of these contractions upon the recti *in vitro*. One or other of the ocular muscles was dissected in the living anaesthetised dog, their

anterior insertions into the sclera being retained, and the muscle bellies being freed to the apex of the orbit where they were separated from their origins. The preparation was immediately transferred into a muscle-bath of Locke's solution maintained at body temperature, and a record of the activity of the muscle taken on the addition of various drugs.\* It will be seen from fig. 9 that on the addition of 0.01 mg. of acetylcholine a short, sharp contraction took place (fig. 9, A). Immediately after reaching the peak of its contraction the muscle commenced to relax, and if the acetylcholine was now washed out of the muscle-bath the muscle rapidly returned to normal. On the addition of a mg. of choline chloride a slower and smaller contraction was registered, which was maintained for a considerable time without falling; immediately the choline was washed out from the bath, however, the curve returned to the normal level (fig. 9, E). If acetylcholine was given and was not removed when the muscle

FIG. 9.—A. At Ac 0.01 mg. acetylcholine; at E 0.1 mg. eserine. B. At Ac 0.01 mg. acetylcholine; at A 0.2 mg. adrenaline. C. At Ac 0.01 mg. acetylcholine; at At 1.0 mg. atropine. D. At Ac 0.01 mg. acetylcholine. E. At C 1 mg. choline washed out at X. F. At Ac 0.01 mg. acetylcholine washed out at X. G. At N 1 mg. nicotine; at Ao 1 mg. acetylcholine. Upper curve, blood pressure; lower curve, muscle record; inset, intra-ocular pressure. Doses added in 15 c.c. bath.

began to relax so that the preparation remained in contact with it for some time, the tracing showed a rapid relaxation until approximately one-half of its original height was reached; thereafter no further relaxation took place, but the muscle remained partially contracted, showing a behaviour exactly similar to that obtained with choline (fig. 9, F). It would appear that in this case the acetylcholine was rapidly destroyed and was converted into choline which exhibited its usual reaction. In comparing the relative effects of choline and acetylcholine in this case, it is interesting that the former does not show the immensely greater activity which it is found to exhibit when its effects on

\* We have to thank Dr. Dale for the opportunity of using his muscle-bath apparatus at the Medical Research Council's Laboratory at Hampstead; and Dr. Gaddum of this Laboratory for his personal assistance in these experiments.

lowering the blood pressure are studied (about 1,000 times as great) (Dale, 1929).

In further experiments 0.1 mg. of eserine was added to the Locke's solution in the muscle-bath, whereupon, as would be expected, the tone of the muscle slowly and gradually increased (fig. 9, A). The addition of 0.01 mg. of acetylcholine in these circumstances showed a contraction very similar to that which had been obtained before (fig. 9, B). The addition of 0.2 mg. of adrenaline to this solution had no effect upon the state of the muscle as registered in the tracing (fig. 9, B), but the addition of 0.01 mg. of acetylcholine thereafter showed that its contractility had been increased very considerably (fig. 9, C). After the addition of 1 mg. of atropine a similar dose of acetylcholine showed no alteration in its effects (fig. 9, D). The addition of 1 mg. of nicotine to the solution at once produced a contraction very similar in type to that caused by acetylcholine, although the muscle did not relax to its former extent: the addition of acetylcholine thereafter was completely without effect (fig. 9, G).

The results of these experiments with the surviving isolated muscle corroborate in every way those obtained in the intact animal. The extra-ocular muscles contract under the influence of acetylcholine, choline and nicotine; the contraction with acetylcholine is uninfluenced by atropine, is increased by adrenaline, and abolished by nicotine.

#### *Discussion.*

The literature on pseudo-motor contraction has been discussed so frequently in recent times that it seems redundant to enter into it fully here. Three sets of circumstances seem to be correlated. In the first place we have already noted that the voluntary muscles of amphibians and birds exhibit a contraction with choline and nicotine, and that, moreover, the foetal muscles of mammals exhibit a similar phenomenon until such time as their motor nerve supply has grown out from the central nervous system to reach them. In the second place, it will be remembered that Vulpian and Phillippeaux (1863) cut the hypoglossal nerve to the tongue (*i.e.*, the motor nerve) and, after it had degenerated, on stimulating the chorda tympani (which is normally a vaso-dilatator), they observed a muscular contraction, a phenomenon which was verified by Heidenhain (1883), who showed that the reaction was not vascular in origin. Again, Sherrington (1894) cut the anterior roots of the nerves to the muscles of the limbs, and after they had been allowed to degenerate, he obtained a similar contraction of these muscles on stimulating the posterior roots. Although much has been written on the subject it would appear that there is no proof

of the nature of the nerve fibres involved ; indeed, a sympathetic, an autonomic, and a somatic origin have been suggested by various writers. Van Rijnberck (1917) showed that the effect was independent of the sympathetic ; while Hinsey and Gasser (1929) have brought forward a large body of evidence to suggest that the fibres concerned are small fibres in the sensory roots which have their cell-stations in the posterior root ganglia and terminate in the perimysial connective tissue and in the adventitia of the blood vessels (Ranson, 1911-29 ; Hinsey, 1923-27). It is probable that these fibres normally mediate vaso-dilatator activity by antidromic responses.

In the third place, Frank, Northmann and Hirsch-Kauffmann (1922-23) found that after the motor nerves had been cut and allowed to degenerate a similar contraction was obtained both in the tongue and the skeletal muscles on the injection of choline ; and their observations have been amply confirmed by Dale and Gasser (1926) and others. It appears, therefore, that sensory nerves, peripheral stimulation of which normally causes only vaso-dilatation, produce a choline-like action in denervated muscle, and that choline, which normally causes vaso-dilatation, excites voluntary muscle which has been deprived of its motor nerves to contraction (Dale, 1929). It thus appears to be the case that the normal (non-denervated) extrinsic muscles of the eye of mammals act, so far as the effect of choline and nicotine is concerned, in a manner exactly parallel to the voluntary muscles of such species below mammalia which have been investigated, or to the other voluntary muscles of mammalia before they have received their motor nerve supply or after when they have been deprived of it.

The problem as to why these muscles show this difference is a difficult one. The sequence—muscles of lower vertebrates, foetal muscles, and ocular muscles—is a suggestive one, and it would seem to indicate that the latter are able to avail themselves of an archaic and primitive mechanism which the other voluntary muscles have lost. It would be difficult to suggest any physiological reason for this peculiarity ; in the present state of our knowledge it would merely seem to add one other unexplained circumstance to the problem of the ultimate source and morphological nature of these muscles.

Apart from the teleological value of the phenomenon, its physiological mechanism presents points of interest. The anomalous physiological behaviour of these muscles suggests an anomalous nerve supply, and of this there is ample evidence. It has long been known that in addition to the motor nerve endings derived from the IIIrd, IVth and VIth nerves, an unusually large number of nerves of the sensory type are met with in the extrinsic muscles of

the eye, a circumstance which it would seem obvious to correlate with their extremely precise postural activity, and the definite spatial sense in visual perception which these muscles appear to possess (Sherrington, 1918). Their endings are epilemmal and include grape-like structures which were early demonstrated by Huber (1899), Crevatin (1900), Dogiel (1906), and others. It is noteworthy that they resemble closely the nerve-endings described by Kulchitsky (1924) in the muscles of the frog (which contract with choline), and there appears to be no similar structure in the limb muscles of mammals. It has been suggested (Bocke, 1927; Woollard, 1927, and others) that these small fibres belong to the sympathetic, an assumption which seems based on little adequate evidence. Where these fibres come from is not known. It might be suggested that they come from the Vth, and, in considering this nerve, one at once thinks of its enigmatic mesencephalic root. The work of Johnson (1909), Kidd (1910-13), and Willems (1911) seems to indicate that this root represents a posterior root ganglion buried within the central nervous system, and although the view that it is proprioceptive in nature subserving the mandibular muscles can be well substantiated, more especially on phylogenetical considerations (Kappers, 1920), and also by the experiments of May and Horsley (1910), yet Winkler (1921), on histological grounds, considered it autonomic in nature subserving vaso-motor reflexes. If the latter view is correct it might conceivably be linked up with the present phenomenon. On the other hand, after section of the Vth nerve Sherrington and Toser (1910) found little or no degeneration in the motor nerves to the muscles; nor could Harrison (1909) find any clinical evidence of such a connection. Moreover, since the action of choline runs *pari passu* with the stimulation of sensory nerves in denervated skeletal muscle, and since choline produces a contraction of the recti without motor denervation, one would expect that stimulation of the sensory nerve involved would give a similar reaction in similar circumstances; and on stimulating the mesencephalic root of the trigeminal, May and Horsley (1910) recorded no muscular contraction, and on stimulating the Gasserian ganglion after proximal section of the Vth and degeneration of the sympathetic, Henderson and Starling (1904) found no rise in the intra-ocular pressure, which excludes any muscular contraction. If these last assumptions are correct, the evidence, although not definitely conclusive, is against the participation of this nerve in the type of activity under consideration.

On the other hand, it would seem much more probable that the phenomenon is in some way related to the nerve fibres which have been demonstrated in the IIIrd, IVth and VIth nerves by Huber (1899), Sherrington and Toser (1910),



and others, and the ganglion cells which have been found in considerable numbers both in their intra-cranial course and in the orbit (Thomson, 1887 ; Nicholls, 1915 ; Tozier, 1912 ; Nicholson, 1924). The histological appearance of these ganglion cells and the evidence of the degeneration experiments of Sherrington and Toser (1910) strongly suggest that these fibres are sensory in type. The IIIrd, IVth and VIth nerves therefore appear to be afferent-efferent in type, differing from typical segmental nerves in that they have no separate ganglionated roots. It is possible, as Edgeworth (1928) suggests, that all the ocular muscles are developed from the præmandibular somite, and that the IVth and VIth nerves are not segmental in nature but are migrated portions of the IIIrd ; but in any event this neuro-muscular complex forms a morphological unit with distinct and separate characters. It seems difficult to dissociate these peculiar morphological characteristics from their peculiar physiological behaviour.

#### *Conclusions.*

1. Choline, acetylcholine and nicotine produce a slow, tonic contraction of the normal extrinsic muscles of the eye. This is the only recorded example of this type of contraction occurring in the non-denervated voluntary muscles of mammals. In this respect the ocular muscles of mammals resemble the voluntary muscles of species below mammalia, or the other voluntary muscles of mammalia before they have received their motor nerve supply or after they have been deprived of it.

2. The contraction is unaffected by atropine, increased by adrenaline, and abolished by nicotine and curare.

3. The morphological basis of this unique behaviour of these muscles is discussed.

It is a pleasure to express our indebtedness to Prof. Lovatt Evans and Dr. H. H. Dale for their advice and criticism in the conduct of these experiments.

The expenses of this research were paid by a grant from the Medical Research Council.

#### REFERENCES.

- Boeke, J. (1927). 'Z. mik. Anat.,' vol. 8, p. 561.  
 Crevatin, F. (1900). 'Rend. Acad. Sci. Ist. Bologna,' vol. 37.  
 Dale, H. H., and Gasser, J. C. (1926). 'J. Pharm. and Exp. Therap.,' vol. 29, p. 53.  
 Dale, H. H. (1929). 'Lancet,' vol. 216, pp. 1179, 1233, 1285.  
 Dogiel, A. S. (1906). 'Arch. mik. Anat.,' vol. 63, p. 501.  
 Duke-Elder, W. S. (1930). 'J. Physiol.' *In the press.*

- Edgeworth, F. H. (1928). 'Phil. Trans.,' B, vol. 217, p. 39.
- Frank, E., Northmann, M., and Hirsch-Kauffmann, H. (1922). 'Arch. Ges. Physiol.,' vol. 197, p. 270.
- Frank E., Northmann, M., and Hirsch-Kauffmann, H. (1923). *Ibid.*, vol. 198, p. 391.
- Harrison, P. W. (1909). 'Johns Hopkins Med. Bull.,' vol. 20.
- Heidenhain, R. (1883). 'Arch. Physiol.,' Suppl., p. 133.
- Henderson, E. E., and Starling, H. S. (1904). 'J. Physiol.,' vol. 31, p. 305.
- Hinsey, J. C. (1923). 'J. Comp. Neurol.,' vol. 36, p. 273; and (1927), vol. 44, p. 87.
- Hinsey, J. C., and Gasser, H. S. (1929). 'Amer. J. Physiol.,' vol. 87, p. 368.
- Huber, G. C. (1899). 'Anat. Anz.,' vol. 15, p. 335.
- Johnson, J. B. (1909). 'J. Comp. Neurol.,' vol. 19, p. 593.
- Kappers, A. (1920). "Vergleichende Anatomie des Nervensystems," Haarlem.
- Kidd, L. J. (1910). 'Rev. Neurol. and Psychol.,' vol. 8, pp. 594, 673, 749.
- Kidd, L. J. (1913). *Ibid.*, vol. 11, p. 507.
- Kulchitsky, N. (1924). 'J. Anat.,' vol. 59, p. 1.
- Langley, J. N. (1906). 'Proc. Roy. Soc.,' B, vol. 78, p. 170.
- Langley, J. N. (1907). 'J. Physiol.,' vol. 36, p. 351.
- Langley, J. N. (1908). 'J. Physiol.,' vol. 37, p. 205.
- Langley, J. N. (1909). 'J. Physiol.,' vol. 39, p. 247.
- Langley, J. N. (1913). 'J. Physiol.,' vol. 47, p. 159.
- Langley, J. N. (1914). 'J. Physiol.,' vol. 48, p. 73.
- May, O., and Horsley, V. (1910). 'Brain,' vol. 33, p. 175.
- Nicholls, G. E. (1915). 'Proc. Roy. Soc.,' B, vol. 88.
- Nicholson, H. (1924). 'J. Comp. Neurol.,' vol. 37, p. 31.
- Ranson, W. (1911). 'Amer. J. Anat.,' vol. 12, p. 67.
- Ranson, W. (1929). 'Arch. Neurol. and Psychiat.,' vol. 23, p. 265.
- Reisser, O. (1921). 'Arch. Exp. Path. Pharmacol.,' vol. 91, p. 343.
- van Rijnberck, G. (1917). 'Arch. Néerl. Physiol.,' vol. 1, p. 257.
- Rückert, W. (1930). 'Arch. Exp. Path. Pharmacol.,' vol. 150, p. 221.
- Sherrington, C. S. (1894). 'J. Physiol.,' vol. 17, p. 253.
- Sherrington, C. S. (1918). 'Brain,' vol. 41, p. 332.
- Sherrington, C. S., and Toner, F. (1910). 'Proc. Roy. Soc.,' B, vol. 82, p. 450.
- Thomson, R. (1887). 'Virchow's Arch.,' p. 109.
- Tozier, F. M. (1912). 'J. Physiol.,' vol. 45.
- Vulpian, A., and Phillipeaux, J. N. (1863). 'C. R.,' vol. 61, p. 1009.
- Winkler, C. (1921). 'Opera Omnia,' vol. 7.
- Willems, E. (1911). 'Le Névrase,' vol. 12, p. 5.
- Woodard, H. H., 'C.R. Assoc. Anat.,' 1927.

## *Secondary Electronic Emissions from Metal Foils and Animal Tissues.*

By W. V. MAYNEORD, M.Sc.

(Communicated by H. Hartridge, F.R.S.—Received March 26, 1930.)

(*Extract and Summary.*)

### *Experiments with Animal Tissues.*

We wish finally to describe experiments in which an attempt was made to make measurements with an ionisation chamber, one side of which consisted of animal tissues. The physical aspects of the results only will be discussed.

The graphited paper chamber which previously served as standard was here abandoned as still showing too great variation compared to a standard air chamber to act as the standard of comparison against tissues, since the latter were themselves likely to be approximately "air" substances. We have therefore, constructed a small cylindrical chamber of graphited cellophane, 14.7 mm. long, 12.7 mm. diameter, having walls 0.002 cm. thick. The chamber is sealed with a thin celluloid end, the cylindrical portion being made by wrapping the graphited cellophane around a brass former and sealing with a saturated solution of cellophane in amyl acetate. This chamber was tested against the air chamber as before and it will be seen that from 60 K.V. with 0.5 mm. Al filtration to 130 K.V. and 10.7 mm. Al filter, the deviation is not greater than the experimental error of 2 per cent.

Peak voltage.	Filter.	Ratio.	Peak voltage.	Filter.	Ratio.
KV.	mm. Al		KV.	mm.	
60	0.5	1.67	130	0.5	1.69
80	0.5	1.70		1.0	1.69
100	0.5	1.69		2.0	1.66
120	0.5	1.69		4.0	1.70
130	None	1.62		6.0	1.68
				10.7	1.69

During preliminary experiments it was soon found that the introduction of wet fresh animal tissues into an ionisation chamber quickly ruined the insulation of the gold leaf system but that dried tissues could very easily be used. Moreover, the dried tissues caused very definite divergences between the two ionisa-

tion chambers. We will here, however, describe the results of a set of recent experiments in which the difficulties were overcome and experiments made with wet tissues.

The ionisation chamber (fig. 1) consists of a carbon ring A on to which is

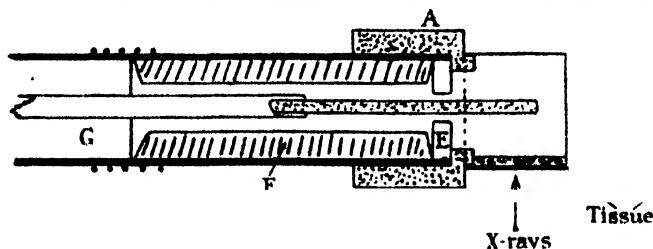


FIG. 1.

fixed a graphited celluloid cube B having one side and base removed. The animal tissue lies on a strip of graphited celluloid L which is itself held in position by means of a second strip bent round the ionisation chamber and having small flanges turned outward. The central carbon rod of the ionisation chamber passes into the chamber through a hole in a celluloid disc E 2 mm. thick, the hole being sufficiently large to allow the rod to pass without touching the celluloid; 1 mm. clearance is allowed. The rod is insulated by means of amber at G, the insulation being thus far removed from the chamber, while around the outside of the brass cable carrying the amber and carbon ring, a small heating coil ("Pladuram" wire 0.117 mm. diameter) is wound which enables the surface of the insulating material to be kept at a slightly higher temperature than the chamber itself. The brass cable is protected with lead F, 3 mm. thick, so as to avoid stray ionisation in the air, the volume of air being also reduced to a minimum. It was found that in this way the introduction of wet substances into the ionisation chamber caused no appreciable natural leak to develop. A number of preliminary experiments were performed. It was first verified that the heating of the apparatus caused no appreciable variation in ionisation current in the chamber.

In these experiments each ionisation chamber, the standard cellophane and the tissue chamber, had separate insulating cables of different lengths and therefore capacities. These cables were led to a specially constructed mercury throwover switch by means of which either chamber could be connected to the gold leaf electroscope, the switch being carefully electrostatically shielded and situated near the electroscope. The observer could, therefore, without disturbing the arrangement, make readings with cellophane chamber and tissue chamber alternately. This procedure was adopted throughout, the mean of

three readings of each chamber being taken, the readings of the two chambers being made alternately. In order to find the effects of the differences of length of cables, as nearly as possible similar cellophane chambers were fitted to each cable. For a number of equivalent wave-lengths the ratio of the ionisation currents observed with the two chambers side by side was obtained. The ratio was independent of the wave-length and equal to 0.77. The tissue chamber now replaced one of the cellophane chambers, the base of the chamber being constructed of graphited cellophane in place of animal tissue. The ratio, corrected for the difference in volume of the tissue chamber when containing the tissue on top of the cellophane, was 0.78. Thus any deviation subsequently observed could not be due to the celluloid or other materials used in the construction of the tissue chamber.

It is clear that we would expect theoretically the animal tissues to be equivalent to a cellophane wall for very short wave radiations, say for  $\gamma$ -rays. The mean of the ratios of the ionisation currents observed in the cellophane and tissue chambers for  $\gamma$ -rays was 0.78, all types of animal tissue agreeing with this value within the limits of experimental error.

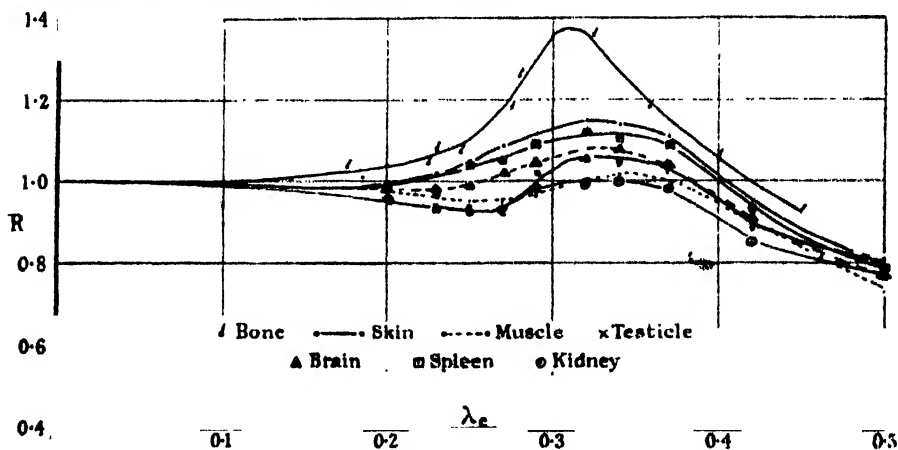


FIG. 2.

All experimental results given below have been corrected so as to give the value 1.00 throughout for an air-like substance, for instance, graphited cellophane.

A healthy rat was killed and immediately dissected, blocks of skin, testicle, brain, spleen, kidney, muscle, heart and liver being taken for examination. Each block of tissue was frozen on to the stage of a carbon dioxide freezing microtome and a section exactly 1 mm. thick was cut. This section was then trimmed to the correct size to cover the floor of the ionisation chamber, trans-

ferred to the celluloid strip and the ionisation currents in the chamber observed simultaneously with the current in the cellophane chamber as described above. The results of one set of experiments are given in fig. 2. The curve for bone was obtained by laying slips of human compact bone, approximately 1 mm. thick, in the position normally occupied by the single slice of tissue.

It will be seen that for short equivalent wave-lengths that the currents in the two chambers do not diverge very greatly from each other, but that for the longer wave-lengths the tissues diverge from strict air equivalence and from each other. The rise in the ratio is presumably to be interpreted as the rise to importance of the fluorescent absorption in the small amounts of heavy elements in animal tissues, but in view of the difficulty of estimating the relative importance of the wall radiation and the ionisation in the air itself we cannot at the moment carry the theoretical consideration further. The subsequent fall in the ratio, is, as in the case of the foils, to be interpreted as the results of absorption in the walls of the chamber and decreasing penetration of secondary electrons. It appears, however, that for long wave radiations ( $\lambda_c = 0.3 \text{ \AA}$ ) a standard air wall ionisation chamber would underestimate the energy absorption in an animal tissue, but appears to be quite suitable for very short wave-length radiations. Further experiments in order to obtain more quantitative information are proceeding.

#### *Summary.*

The paper describes experiments on small ionisation chambers artificially made sensitive to different X- and  $\gamma$ -radiations by the insertion of foils of different elements. It is shown that compared to an air chamber these ionisation chambers show a maximum sensitivity in the region of medium wave-lengths. The theory of the occurrence of this maximum is discussed.

A small ionisation chamber suitable for  $\gamma$ -ray measurements is described, and it is shown that the relative intensity of primary and secondary scattered beams of rays was differently estimated according to the materials of which the chamber was constructed.

The experiments were extended to include the effects to be observed with ionisation chambers containing animal tissues. It is shown that again a region of maximum sensitivity is observed and that various tissues diverge by varying extents from "air equivalence." The significance of these observations is briefly discussed.

[The full paper is printed in 'Proceedings,' Series A, vol. 130, pp. 63-80.]

*Address of the President, Sir Ernest Rutherford, O.M., at the  
Anniversary Meeting, December 1, 1930.*

At this, our Annual Meeting, we are naturally conscious of the severe losses in our ranks in the course of the year. We have to deplore the removal by death of some of the best known and most valued Fellows, including Lord Balfour and Sir William McCormick, elected under Statute 12, Professor Le Bel, Foreign Member, and twelve Fellows of the Society.

The death of the EARL OF BALFOUR at the age of 82 removed from our midst a public figure of the first magnitude. Although Balfour's activities covered a wide field, and although through a great part of his career he carried heavy responsibilities in guiding the affairs of the Nation, science was always with him a topic of primary interest. If he cannot be said to have made original contributions to scientific knowledge himself, there can be no doubt that his championship of the cause of science was of the greatest indirect benefit. As First Lord of the Treasury in 1900, he did much to help forward the scheme for the National Physical Laboratory, in which his brother-in-law, Lord Rayleigh was interesting himself. He was constantly called upon to preside, or to speak, at meetings for the furtherance of scientific objects, or the commemoration of the great scientific careers of the past, and seldom failed to add distinction to such occasions. He may indeed be regarded as a chief interpreter of science to the English public during his generation. He was President of the British Association at Cambridge in 1904. He was elected to the Royal Society under Statute 12, as early as 1888, at the age of 40 years. He served on the Council in 1907-08 and again in 1912-14. But, perhaps, his chief work for science was in his later years, after the most active part of his political career was over. In two successive terms of office as Lord President of the Council, he was the Minister responsible for the Department of Scientific and Industrial Research, and for the Medical Research Council. Of the latter body he acted as chairman until the onset of his illness. He watched the scientific interests under these departments with close personal attention, and did much to establish them on a permanent basis. Finally, to him was due the Committee of Civil Research,

complementary to his older creation of the Committee of Imperial Defence. He was Chancellor of the Universities of Cambridge and Edinburgh, and President of the British Academy. His is a place which will not easily be filled.

Sir WILLIAM McCORMICK, who died suddenly in his seventy-first year, had a very varied career of service to the Universities and to the State in many capacities. At one time Professor of English in University College, Dundee, he became the first Secretary of the Carnegie Trust for the Universities of Scotland. He was for many years Chairman of the University Grants Committee where his personal influence and advocacy were largely instrumental in obtaining the increased grants for the Universities in the difficult post-war period. He was the first Chairman of the Advisory Council of the Department of Scientific and Industrial Research which was set up in 1916 under the pressure of the war to organise the application of science to industry. The success of this Department, which is known to you all, was in no small part due to his financial acumen and organising ability. Although not trained as a scientific man, he had a wide interest in science and sympathy with the scientific outlook and worked loyally with his band of scientific advisors—originally all Fellows of our Society. The Society was glad to recognise the value of his services to Science by electing him a Fellow under the special statute. His charm of manner and breadth of outlook endeared him to all his friends, and he will be greatly missed.

The death of Admiral Sir HENRY JACKSON at the age of 75, has caused deep regret in both naval and scientific circles. When in the Navy, in 1891, he began experiments to test the utility of Hertzian waves as a means of communication between ships at sea, and was largely instrumental in the rapid development of this method of signalling in the Navy. As early as 1902, in a paper to our "Proceedings," he drew attention not only to a failing of signals but also to their reappearance as the distance between the signalling and receiving ships increased. During the war, he was appointed as First Sea Lord in 1915 when Lord Fisher left the Admiralty. On his retirement from active service, he was appointed, in 1920, Chairman of the Radio Research Board, instituted by the Department of Scientific and Industrial Research. In this capacity he did admirable work in encouraging researches on the fundamental problems of radio-transmission. He was elected to our Fellowship in 1901 and was awarded the Hughes Medal in 1926. His charm of manner, simplicity of nature and devotion to duty endeared him to all those with whom he came in contact.



IN PERCY ALEXANDER MACMAHON the mathematical world has lost a distinguished and enthusiastic worker of marked individuality, as well as a most attractive personality. An algebraist of great resource and insight, he was early attracted by the theory of invariants (in the most extended sense) and symmetric functions, and his discoveries in connection with the differential operators in these theories gained at once the warm appreciation of the great exponents, Cayley and Sylvester. By a natural sequence he was led on to intricate problems of Combinations and Probability, to which his researches had already opened a line of attack. His work on these subjects was afterwards collected in the two volumes published under the title "Combinatory Analysis," by the Cambridge Press. An interesting by-product of his later years was the theory of repeating patterns, with which he occasionally amused an audience, and which afforded an outlet for his strong sense of humour. He died on Christmas Day 1929, at the age of 75.

Dr. SEBASTIAN ZIANI DE FERRANTI who died in January last was the great pioneer of electricity supply at high pressure. He was of Italian descent from one of the Doges of Venice, but was educated and lived all his life in England. At the age of eighteen, he patented the Ferranti alternator and a few years later, while still a young man, designed a system of supply from Deptford which was transmitted at 10,000 volts. Little was then known about these high pressures and no instruments for measuring them were in existence. Ferranti consulted Lord Kelvin on some abstruse points in mathematical physics during the course of his work. In 1892, he severed his connection with supply and started the firm of Ferranti, Ltd., which manufactured many of the machines and devices he had perfected. He was careful to make machines which would be safe for the workman to handle and loved to invent devices which lightened domestic drudgery. Ferranti was a great engineer and lived to see his ideas developed and huge high pressure networks constructed in almost every country. Were it not for him the world would be perceptibly poorer to-day. He was a man of great modesty but much personal charm.

The premature death of Professor HUGH LONGBOURNE CALLENDAR has removed from our ranks an experimental physicist of great ability and insight. Elected Fellow of Trinity College, Cambridge, in 1886, he first devoted himself to the development of platinum thermometry as a means of very accurate measurement of differences of temperature. In 1893 he became Professor of Physics in McGill University, Montreal, and was largely responsible for the admirable equipment of the then new Macdonald Physics Laboratory. While in Montreal he began his researches on continuous

electrical calorimetry and had a wide influence in promoting interest in research in that University. After a short term of service in University College, London, in 1902, he was appointed Professor of Physics in the Imperial College of Science and Technology and filled this chair up to the time of his death. An unusually careful and accurate experimenter with marked interest in engineering applications, he devoted himself to accurate measurements of many important quantities in heat, and in this respect was a worthy successor of Regnault. One of his last pieces of work was the preparation and publication of accurate steam tables which have proved of great utility to industry. A man of wide training and all-round capacity, his death has left a gap in the scientific world which is difficult to fill.

EDWIN TULLEY NEWTON, who died in his ninetieth year, was for many years a leader in the study of fossils in Great Britain. Interested in natural history, he attracted the notice of Huxley and became his assistant. In 1882 he was appointed palaeontologist to the Geological Survey and held this post until his retirement in 1905. Some of his most fundamental contributions to vertebrate palaeontology are contained in three memoirs published in our "Transactions." He was elected to our Fellowship in 1893 and was awarded the Lyell Medal by the Geological Society.

MR. ALAN ARCHIBALD CAMPBELL SWINTON was a member of our Council at the time of his death. Trained as an electrical engineer he took an active part in the development of the electrical industry both as consulting engineer and as a director of many companies. A man of wide scientific interests he was one of the first to obtain X-ray photographs in this country, and was much interested and helpful in the early development of experiments on radio-communication. Of wide reading and sympathies, he was well known to scientific men and was a familiar figure in scientific gatherings. He was most interested in the work of the Society and served on a number of its committees as well as on the Council. The Society is much indebted to him for a generous gift of £1,000 in 1926 to form the nucleus of a General Purposes Fund.

KENNETH JOSEPH PREVITÉ ORTON passed away on March 16 last in his fifty-ninth year. After his student days at Cambridge, Heidelberg and London, he joined the chemical staff of the Medical School at St. Bartholomew's Hospital, and in 1903 became Professor of Chemistry in the University College of North Wales at Bangor. Orton carried out original work on the mechanism of organic chemical reactions and developed more particularly the study of the chlorination of aromatic compounds; he was one of the first systematically to apply

physico-chemical methods to the study of organic reactions. Orton was a vigorous and refreshing personality with many interests lying far outside his particular branch of chemistry; he was an experienced field geologist and ornithologist. He devoted himself with enthusiasm to the development of the University of Wales and his early death leaves all who are interested in Welsh education under a keen sense of loss.

JOHN OLIVER ARNOLD, who died on March 27 last, at the age of 71, was closely identified with the progress of the metallurgy of steel. Appointed while a young man to the Professorship of Metallurgy in the Sheffield Technical School, he saw the growth of that institution into the Applied Science Department of a University, and established a connection with the steel industry which has had lasting effects. It is largely due to his efforts that scientific control has been so generally adopted in metallurgical works, whilst his own researches did much to forward the application of metallographic methods, originally due to his fellow townsman, Sorby.

The death of JOSEPH ACHILLE LE BEL on August 6 last, takes from among us one of the veterans of French chemical science. In 1874, simultaneously with, but independently of, van't Hoff, Le Bel enunciated the doctrine of the asymmetric carbon atom and thereby laid the foundation of modern stereochemistry; it would be difficult to over-state the part which this fundamental theoretical conception has played in the development of structural organic chemistry. Le Bel, who was born in Alsace in 1847, received our Davy Medal in 1893, and was elected a Foreign Member of the Society in 1911. In 1925 he made a gift of money to the Society with the request "that you subsidise research rather than subsidise students, for I think we have plenty and to spare of savants who have fattened on examinations, but not enough of the people who employ their time in solving problems of interest. Hitherto the Royal Society seems to me to have used its money well. I hope that it will continue."

HERBERT HALL TURNER, who died suddenly at a meeting of the International Union of Geodesy and Geophysics, was Savilian Professor of Astronomy at Oxford. In 1893, when photography was beginning to be applied to the determination of positions of stars, he introduced the methods of reduction which have been employed with little modification ever since. He devoted himself very energetically to the project of the International Astrographic Catalogue, and having completed his own (Oxford) section in good time, rendered assistance to other observatories which were behindhand. He was also interested in variable stars and seismology, both of which subjects gave scope for his favourite

theme—harmonic analysis and the search for periodicities. He performed still greater service to astronomy by the infinite pains he took to assist and encourage the amateurs and the younger men and to keep in touch with those working in isolated observatories in the Dominions and elsewhere. A very ready speaker, a genial chairman and leader in any kind of activity, he will be greatly missed at astronomical meetings and conferences.

HAROLD BAILY DIXON entered Oxford with a classical scholarship in 1871, but soon turned his attention to science under the influence of Dr. A. Vernon Harcourt; in 1876 he commenced those experimental studies on gaseous explosion with which he was occupied until the morning of his death. During his early work he showed, contrary to the conclusions arrived at by Bunsen, that the explosion of gaseous mixtures is governed by Berthollet's "law of mass action"; he also discovered that carefully dried mixtures of carbonic oxide and oxygen cannot be ignited by an electric spark, and so opened up a new and fertile field of investigation which is still under exploration. Dixon carried out a large amount of difficult but very precise work on the high speed of travel of the explosive wave in gaseous mixtures and was the first to make careful measurements of the ignition temperature of such mixtures. In 1886 he was appointed to succeed Sir Henry Roscoe as Professor of Chemistry in Owens College, Manchester, a position which he held until 1922, when he was elected Honorary Professor in the University of Manchester. Although Dixon was intensely occupied throughout his life with scientific work of fundamental importance and far-reaching consequences, he devoted himself with enthusiasm to furthering the academic, social and athletic activities of his University. In this, his administrative gifts, his brilliance and felicity as an exponent, and his power of arousing enthusiasm in his students, made him again a commanding figure. As one of the foremost authorities on gaseous explosion, Dixon was frequently called upon to assist in Government enquiries into coal-mine disasters. He was elected to the Society in 1886 and was awarded a Royal Medal in 1913; he delivered the Bakerian lecture on "The Rate of Explosion in Gases" in 1893. On September 18 last he passed away suddenly in his seventy-ninth year, whilst retaining unimpaired until the end those mental gifts which so many of us have been privileged to admire.

WILLIAM DILLER MATTHEW, Professor of Palæontology in the University of California, was born in Canada, and though domiciled during the whole of his scientific career in the United States of America, retained the nationality of his birth. For thirty years he was associated with Dr. Henry Fairfield Osborn, in the charge of the great collection of fossil vertebrates in the American Museum

of Natural History, New York, to which collection Matthew's own discoveries in the Western States greatly contributed. In 1927 he accepted the chair in California, with the expectation of a long period yet before him for further work on the fossils of far Western America. His investigations and writings have played a large part in the development of knowledge of the extinct vertebrates and theories concerning their evolution, and the science of palæontology has suffered a heavy loss by his death at the early age of 59 years.

Dr. JOHN WILLIAM EVANS, who was admitted a Fellow of the Society in 1919, was a geologist of wide and varied experience. He had carried out important geological investigations in Southern and Western India, and in South America, besides occupying himself with many problems in theoretical geology. Of late years he had given much attention to the application of geographical methods to the study of the earth's crust.

I shall now pass to a discussion of some matters of general interest to the Society. In the Report of the Council for last year, it was announced that the Committee, which was appointed by the International Research Council to revise the statutes now in force, had adopted all the suggestions which the Council of the Royal Society had made. Since then the Committee's Report has been considered by the Executive Committee of the Research Council which has adopted it after making a few modifications and has recommended these revised statutes to the General Assembly for approval at its meeting in July, 1931. The changes introduced are all in the direction of giving to the Unions greater freedom in arranging their own affairs, the Council performing the function of a co-ordinating body. The Unions will be members of the International Council and will have representation in the General Assembly at its triennial meetings. The new Statutes are such as should facilitate that international co-operation in science which it is the aim of the organisation to promote.

At these anniversary meetings the President has frequently taken the opportunity of putting before the Society some recent and important development of science within the scope of his own personal interest. To-day, on this last occasion of my addressing the Society from this chair, I have felt rather disposed to look back over the whole term of my Presidency, and into that of my immediate predecessor, and to put on record some of my impressions of the work of the Society and of the way in which it is responding to the new opportunities for the promotion of science which have come to it in this present period.

I propose, then, to review briefly some aspects of the history of our Society since the end of the war, and to point out the way in which the responsibilities and work of the Society have increased during this period. While in the course of its long history, the Society has received numerous bequests for scientific and general purposes, shortly after the end of the war the funds at its disposal were greatly increased by the receipt of four major benefactions. I refer to the bequests of Miss Lucy A. Foulerton in 1919, of the late Dr. Rudolph Messel, F.R.S., in 1921, of the late Dr. Ludwig Mond, F.R.S., in 1923, and the notable benefaction of our valued friend Sir Alfred Yarrow, F.R.S., in 1923. Miss Foulerton's bequest was made specifically for the promotion of research in the Medical Sciences, while the terms of those by Dr. Messel and Dr. Mond and of the gift from Sir Alfred Yarrow allowed the Society a wider discretion to utilise the interest on the resulting funds for the general object of our Society, namely, the "improving of natural knowledge." Taking these benefactions as a whole, they provide resources for the promotion of research in practically the whole range of the natural sciences which our Society represents.

Under the Presidency of Sir Charles Sherrington, the Council considered with great care the best method of employing these new resources and decided to apply a large part of the incomes of the Foulerton and Yarrow funds in the first instance for the institution of Research Professorships. The first holder of such a Royal Society Professorship was the late Prof. E. H. Starling, appointed Foulerton Professor in 1922. A second Foulerton Professorship was created in 1926, Prof. A. V. Hill being appointed. On the death of Prof. Starling, a Foulerton Professorship was awarded to Dr. E. D. Adrian in 1929. In 1923, Prof. A. Fowler and Mr. G. I. Taylor and in 1924, Prof. O. W. Richardson were appointed Yarrow Professors of the Royal Society.

The Council further adopted regulations for the Messel and the Mond funds, subject to periodical review, which provided for the eventual use of these funds for the support of further professorships as the need and opportunity might arise. Wisely, as I think, they and their successors were content to watch for a time the general effect on scientific progress of the Foulerton and Yarrow appointments, holding meanwhile in reserve the similar opportunities which the Messel and Mond funds would enable them to offer. My close personal contact with him and his work enables me with special pleasure and warm approval to announce that this year the Council have appointed Dr. Peter Kapitza, F.R.S., Fellow of Trinity College, Cambridge, to a "Messel" Professorship of the Royal Society. I

shall have occasion later in my address to refer to the important researches on which Dr. Kapitza has been engaged in recent years, and to the arrangements made with the University of Cambridge for the provision and upkeep of a laboratory suitable for his investigations.

In my address to the Society in 1928 I reviewed briefly the work of the Royal Society Professors up to that date. As I then pointed out, we have been fortunate in securing the services of a group of men of marked research ability. There has been an output of work of high quality and importance, and I am sure we can all agree that this new experiment of endowing research professorships has proved an unqualified success. In all cases, the holders of our professorships have been heartily welcomed by the Universities and Institutions with which they were associated, and they continue to carry out their investigations under excellent conditions. I think there can be no doubt that the appointments so far made have not only added materially to the strength of the research side of the universities concerned, but have led to a marked increase of the research power of the nation.

While our Professors have been allowed a wide discretion in the work they perform, and in many cases direct the work of research schools, and give advanced courses of lectures, there is undoubtedly a general opinion that, at the present time, it would not be wise to increase unduly the number of our Research Professors. There is always the danger that any substantial increase in the number in the near future might lead, in a sense, to the segregation of some of the more vigorous elements in the research life of our Universities from that intimate contact with students and inspiration of their work which, in the case of many investigators of the highest rank, is an essential part of their contribution to the advancement of Science. In instituting these Professorships, the Society has been engaged in a novel experiment and, successful though the result has been, it is still desirable that we should proceed with caution and reconsider our policy from time to time as our growing experience suggests.

In addition to the six professorships which are supported from the trust funds obtained since the war, the Society now offers for the encouragement of younger research workers eight Studentships and Fellowships, namely, the Sorby, and Smithson Fellowships and the Foulerton, the Mackinnon, two Moseley, the Tyndall, and the Lawrence Studentships. The second Moseley Studentship has been established during the year in consequence of the augmentation of the Moseley fund by a bequest from the late Mrs. Sollas.

These Fellowships and Studentships are all financed from funds left to the Society for the specific purpose of their creation. In my address last year, I referred to the institution of a new Research Fellowship, financed from the bequest left by the late Mr. E. W. Smithson. The first appointment to this Fellowship has now been made and I shall say a few words later on the work of the new Fellow.

Taking into consideration the provision now made for the support of research, to mention only some of the major sources, by grants from the large Government funds administered by the Department of Scientific and Industrial Research and the Medical Research Council, by the studentships offered by the 1851 Commissioners, and by the Fellowships for research in the Medical Sciences created by the Beit Memorial Trustees, it is clear that financial support is now available from a number of different sources, for young research workers of promise in this country.

As I have pointed out, the funds held in trust by the Society for the furtherance of research in various branches of science have been very greatly increased during the past ten years and now amount to more than £600,000. The major increase occurred within the short period of five years from 1919 onwards, and the Council, fully conscious of its responsibility on behalf of the Society, in providing for the use of these great resources for the advancement of science, proceeded as I have indicated, with proper caution. After the appointment of the five first research professors, and the creation of the studentships for which certain bequests were specifically made, the Council had still a substantial margin of income from some of the larger funds, and this has been invested, while plans for its use to the best purpose were being matured. In this way about £72,000 has been added to capital, while in each year a considerable sum is still being received in dividends which has not yet been definitely allocated to any special research. Another reason for an initially cautious policy in expending these trust funds, was the difficulty of foreseeing the financial commitments of the Society due to its already existing activities. For example, the rapid increase of the volume of the Society's publication after the War, and the disproportionate rise in its cost, involved an increasing call on the funds available for this purpose. The Council at that time considered that one of the greatest services which they could render to Science, with the funds at their disposal, was to facilitate the publication of the results of research at a cost rendering them accessible to the widest range of scientific workers. The Council to-day have not moved from this opinion, but they have been able better to survey the Society's policy in relation to that of the scientific world in



general, and to estimate more clearly the probable needs of the future. It now seems unlikely that there will be any substantial increase in the amount of publication during the next few years. Moreover, the Council during the present year have given careful consideration to the cost of publication and have decided on an increase in the price of the Society's 'Proceedings' and 'Transactions' to external subscribers. Though the price is still low in comparison with that of other scientific publications and in relation to the increased cost of production, the change should result in the release of a substantial sum which can be allocated for other purposes.

It would, therefore, seem that the time has arrived when we may with prudence consider how some at any rate of the accumulated income from our trust funds may be best expended in promoting some form of scientific research.

Experience has shown that the encouragement of research by minor grants for special apparatus and material is in reasonable measure provided for by the Government Grant, supplemented from the Society's own research funds. The grants to individual investigators from such sources are usually small but suffice to assist materially important researches of a limited scope.

The situation, however, is very different when we consider large scale investigations of a pioneering character, which may require considerable financial support extending over a period of years in order to provide the necessary apparatus and technical assistance to bring the investigation to a definite conclusion. Few of our Universities or other Scientific Institutions are sufficiently well endowed to support large scale researches of this kind, even when the research appears of marked promise and when the idea and the man are forthcoming. In considering the best method of utilising the balance of the Society's present resources, the Council decided that it could best help the advance of science by assisting major researches of this character, and after careful consideration, were impressed with the fundamental importance of the researches at present being carried on by Dr. P. Kapitza, at Cambridge, and the need for continuing this work on a more permanent basis.

It may be helpful at this stage to give a brief history of the origin and development of the work on which Dr. Kapitza has been engaged for the past eight years. Trained as an electrical engineer, he was lecturer in Physics in the Petrograd Polytechnical Institute during 1918-1921. In 1921, he came to England and commenced research work in the Cavendish Laboratory, Cambridge. In 1922, he began experiments to test the possibility of

obtaining intense magnetic fields by sending very strong currents through a coil for such a short interval that the heating effect in the coil is restricted to a permissible value. With the assistance of a grant from the Department of Scientific and Industrial Research, special accumulators were constructed to give the necessary intense currents for a short interval of about  $1/50$  of a second. In this way, fields up to 200,000 gauss were obtained, and it was found practicable to carry out experiments by this method, for example on the Zeeman effect and on the deflexion of  $\alpha$ -particles. In order to carry these experiments still further, it was necessary to have a method of obtaining currents still larger and more under control. For this purpose, a generator of special design was constructed which gives, on short circuit, a current of about 70,000 amperes. The heavy current from the generator is passed for about one-hundredth of a second through a coil and is then broken by means of a specially designed automatic break. The Department of Scientific and Industrial Research gave a very substantial grant for the construction of this apparatus, while Sir William Pope kindly provided a temporary laboratory to instal the plant and to carry out the experiments. In 1926, the laboratory was opened formally by the late Lord Balfour, then Lord President of the Council, who had throughout taken an active interest in promoting these large scale experiments. This pioneering investigation, which was carried out in connection with the Cavendish Laboratory, was only made possible by the generous and bold support of the Department of Scientific and Industrial Research, which, up to the present, has defrayed the complete cost of the apparatus and of the subsequent investigations.

One of the chief difficulties in these experiments has been to construct a coil strong enough to withstand the enormous disrupting forces which arise when a large current is passed through the coil. A number of coils have been constructed which give magnetic fields of between 300,000 and 400,000 gauss over a volume of about 3 c.c. There appears to be no inherent difficulty why fields of the order of 1 million gauss should not be obtained, when called for, by this method. As the current through the coil only lasts for about  $1/100$  second, oscillograph methods are used to determine the strength of the current and magnetic field and to follow the changes in the properties of the material under investigation. There is no special difficulty in conducting experiments with these momentary fields. In fact, a single photograph, obtained in  $1/100$  of a second, may give a complete quantitative record of the magnetic effects produced in a material over a wide range of magnetic field.

The application of these new methods of producing intense magnetic fields

opens up a wide field of research where all magnetic properties can be examined in fields 10 to 30 times greater than those hitherto available by the use of electromagnets.

As soon as the apparatus was in working order, experiments were begun by Dr. Kapitza to investigate the change of resistance of crystals of bismuth in these intense magnetic fields from atmospheric temperature to that of liquid air. This was followed by an extensive investigation of the behaviour of a large number of metals under corresponding conditions. In general, it was found that the change of resistance was at first approximately proportional to the square of the magnetic field, but above a certain critical field, which varied from metal to metal, the change of resistance tended to become linear. On the basis of these new results, he has suggested a new way of looking at the phenomena which underlie the electrical conductivity of metals and its variation with temperature. Preliminary experiments have also been made on the action of these strong fields on the paramagnetism and diamagnetism of certain substances, while a new and sensitive apparatus has been constructed to study magnetostriction effects. An account of the apparatus and the experimental methods, together with the results of some of these investigations, has been published in our 'Proceedings.'

Magnetic phenomena are shown in their simplest form at very low temperatures when the complications due to the motion of the atoms and molecules are largely avoided. In order to obtain temperatures still lower than that of liquid air, a liquid hydrogen plant has been installed during the present year, and is now in working order. Preliminary arrangements have been made to instal a liquid helium plant when this is required for the investigations.

The grant given by the Department of Scientific and Industrial Research for carrying out these researches expires in a few years, while the laboratory temporarily lent for the purpose of these experiments is now required by the Chemical Department. The Department of Scientific and Industrial Research, by its broadminded and far-seeing action, has done a great service to science in thus supporting, through their initial stages, investigations having no obvious or immediate application in practice or industry. Their support for an indefinite further period, however, could hardly be part of the Department's policy. On the other hand, it appeared to the Society's Council, that investigations of this kind, in which new fields of knowledge are being opened up by new methods, had a peculiarly strong claim for support from those funds which they were holding ready for the furtherance of fundamental researches in pure science.

After full consideration, therefore, the Council, in addition to appointing Dr. Kapitza to a Messel Professorship, agreed to offer the University of Cambridge the sum of £15,000 for the building of a suitable laboratory within the next three years, provided the University was prepared to offer an appropriate site and to defray the running expenses of the new laboratory. Negotiations have taken place with the University of Cambridge and, though the matter has not been formally sanctioned by the University, the Council of the Society has received assurances which make them confident that the offer will be accepted and that the University will provide the necessary funds to carry out the work. If the University of Cambridge concurs with these proposals, the Royal Society will thus have been instrumental in founding a new and up-to-date laboratory, primarily designed for carrying out researches in intense magnetic fields, but at the same time providing the essentials of a modern Cryogenic Laboratory for the study of magnetic and other effects at the lowest attainable temperatures.

It is proposed that a Committee should be appointed, which would be responsible for the direction of the work of the Laboratory. The name of the laboratory has not yet been settled, but it would clearly be appropriate if it indicated the connection with the Royal Society and with the late Dr. Ludwig Mond whose bequest furnished the income from which the cost of the laboratory will be defrayed. It should be noted that among the purposes indicated in the will of Dr. Mond for the use of his bequest was "erecting new laboratories."

It will be remembered that, thirty years ago, this country was pre-eminent in the study of effects produced on matter by the low temperature produced with the aid of liquid hydrogen. It will be recalled that the late Sir James Dewar, with the technical assistance of Mr. Lennox, first produced liquid hydrogen in quantity in the Laboratories of the Royal Institution in 1898, and in 1899 the first solid hydrogen was obtained. It was as early as 1893 that Dewar devised the vacuum flask which has proved to be of such fundamental importance in the technique of low temperatures and has so greatly simplified the handling of liquid gases. It is of interest to note that it was decided in 1902 to construct a liquid hydrogen plant, of capacity of about 5 litres of liquid hydrogen per hour, as a British Government exhibit to the St. Louis Exposition in 1904. This plant was placed in the competent hands of Mr (now Sir) Joseph Petavel and I well remember the interest of his demonstrations of the properties of liquid hydrogen at that Exhibition. Some time later, a small liquid hydrogen plant was installed by Dr. Travers, of University

College, in the Laboratory of the late Sir William Ramsay. In the meantime, an efficient Cryogenic Laboratory had been established at Leyden, under the direction of the late Professor Kamerlingh Onnes, For.Mem.R.S. All of you are aware of his success in liquefying helium and of the wide range and importance of the investigations carried out on the effects of low temperatures on the properties of matter. It was only a few years ago that Prof. Keesom, who followed Onnes in the charge of this Laboratory, was successful in producing solid helium.

A few years ago, owing to the energy and enthusiasm of Professor J. C. McLennan, F.R.S., liquid hydrogen and helium plants were installed in the University of Toronto, and have proved their utility in a number of important researches. In recent years modern equipment for the liquefaction of hydrogen and helium has been installed in the Reichanstalt, Berlin, by Dr. Meissner, and very valuable results have been already obtained. Dr. Franz Simon, of the University of Berlin, obtains the temperature of liquid helium by an ingenious method involving the use of liquid hydrogen and the absorption of helium gas by charcoal.

I am sure it will be gratifying to the Society to know that we may soon expect to have an up-to-date Cryogenic Laboratory on a small scale in this country, and thus to take part again in the exploration of this important field of enquiry.

I should emphasise the point that the Council in deciding to incur the liability of the expense of this new laboratory have utilised the income alone of certain trust funds, while the capital of each remains intact. There is still a substantial amount of income from these Trust Funds to be allotted, in accordance with the general aim adopted and kept in view by the Society's Council, for the furtherance of fundamental research in pure science. The policy which they may be expected to maintain in the future, is to keep watch over the whole field of scientific activity, in readiness to give help where there is promise of an important advance, and where the right man for its conduct is available. The Council look with confidence for help and support from the whole body of Fellows in the important responsibilities which they have accepted and have still to undertake on behalf of the Society.

I believe that it is in helping such important schemes of research that the Society can best utilise any research funds which it already possesses or which may become available in the near future. It not infrequently happens that a promising line of research or the development of a new method may be held up or abandoned because of the difficulty of obtaining adequate financial

support. In some important directions, advance can only be made with the help of technical assistance in the construction and use of special apparatus, in some cases on an almost engineering scale.

By its constitution, the Society is especially well fitted to advise and support investigations of this kind with a minimum of that bureaucratic control which is generally so distasteful to the original investigator. While with our modest funds, we can only hope to support a few of such undertakings, I am sure that if more funds were needed for such an important purpose they would soon be forthcoming. This extension of the activities of the Society, whereby it is taking an active and essential part in advising and assisting in the development of fundamental science in this country, cannot fail to have a vitalising effect not only on scientific workers in general, but on the Society itself. Along such general lines, it is not difficult to foresee that the Society will exert an ever increasing influence on the progress of science and thus promote still further the original intentions of its founders.

I referred earlier in my address to the institution by the Society of the *Smithson Research Fellowship*. The Society was fortunate in obtaining applications for this Fellowship from a number of distinguished investigators in different branches of Natural Science. The Selection Committee, consisting of representatives of our Society and of the University of Cambridge, did not find it an easy task to make a selection from among such an able group of investigators.

The first award of the *Smithson Fellowship* has been made to Dr. P. D. F. Murray. After a distinguished undergraduate career in the University of Sydney, Dr. Murray spent two years in research work in the Department of Comparative Anatomy at Oxford, and since 1926, has been lecturer in Zoology at the University of Sydney. Nearly all his work has been in the field of experimental embryology, and he has investigated with conspicuous success, the factors which determine the differentiation and shaping of the limbs and other parts of the body, mostly by the method of transplanting small portions of early embryos on to the chorio-allantoic membrane, where their development proceeds, apart from the influence of the other tissues of the embryo. By this procedure and by the method of tissue culture, Dr. Murray proposes to examine the cellular differentiation of the developing chick, which underlies the coarser morphology. He will work in the first instance at the *Strangeways Research Laboratory* in Cambridge.

Mr. A. H. White, for so many years in charge of the Library of the Society,

has retired this year on pension. Many of our Fellows and particularly our senior Fellows, are well aware of his devoted work in the interests of the Society for such a long period, and of his unrivalled knowledge of the Society's books and history. In recognition of these services, on his retirement, Mr. White has been given the title of Consulting Librarian to the Society. He has been succeeded in his office by Mr. R. Winckworth.

Before I pass on to the presentation of medals, my last official act before the termination of my Presidency, I should like to express my gratitude to the officers and Council of the Society for the consideration and kindness they have uniformly shown me. My path has been made easy by the very efficient help that I have received both from the present officers and those who have retired during my term of office. I would like also to express my thanks to Mr. Towle and the other members of the staff for their unvarying help and kindness. I can assure my distinguished successor from my own experience that he will find his work made pleasant by their ministrations. It has been a great honour for me to preside at your counsels and gatherings, and I can assure you that I have been happy to serve the Society to the best of my powers. I thank you all for this great opportunity and privilege of service.

The Copley Medal is awarded to Sir William Bragg.

To the rapid advance of Experimental Physics in the last thirty years, Sir William Bragg has made conspicuous contributions by his pioneering researches in Radioactivity, X-rays, and Crystallography. When Professor in the University of Adelaide, he was the first to realise, in 1904, the characteristic difference to be expected in the nature of the absorption of the massive  $\alpha$ -particle and the light  $\beta$ -particles expelled from radioactive substances. His experimental researches brought out clearly the rectilinear path of the  $\alpha$ -particles and their limited range of travel. In collaboration with his students, he examined in detail the variation of the ionisation of the  $\alpha$ -particle along its path and its absorption by different kinds of matter. In his researches in X-rays and  $\gamma$ -rays, he was impressed by the difficulty that these high frequency radiations behaved like projected corpuscles—a difficulty which has only been in part resolved to-day. Following the discovery by Laue of the diffraction of X-rays by crystals, he was the first to develop a method for showing that ordinary X-radiation gave bright lines superimposed on a continuous spectrum. This reflection method of studying the spectrum of X-rays has proved of great importance to the development of knowledge. In the hands of Moseley, it supplied a means of showing that the

atoms have all a similar structure and that their properties are defined by a whole number. In the hands of Sir William Bragg and his son, Professor W. L. Bragg, it has provided a powerful tool for unravelling the structure of crystals. In this important development, which has added widely to our knowledge, Sir William Bragg has taken an active part, not only by his own researches, but by the direction of an important school of research on this subject at the Royal Institution. His work throughout is characterised not only by simplicity and elegance but by a clear grasp and exposition of the essential principles involved.

The Rumford Medal is awarded to Professor Peter Debye.

To Professor Debye are due many important advances in the field of heat and radiation. He introduced and developed a theory of the specific heats of solids which is of fundamental importance. By it, for the first time, the main phenomena relating to specific heats and their variation with temperature were quantitatively explained. He made important contributions to the theory of the scattering and reflection of X-rays. Independently of Compton, he put forward the quantum theory of the change of frequency due to the scattering of X-rays—the Compton effect. He was one of the inventors of the powdered crystal method of X-ray crystal analysis. By his introduction of the idea of spatial quantization and by his investigations relating to the electric and magnetic properties of molecules he did much to advance our understanding of radiation and molecular phenomena. In collaboration with Hückel, Debye has developed a theory to account for the properties of strong electrolytes which has many important applications.

A Royal Medal is awarded to Professor Owen Willans Richardson, F.R.S.

In his earlier work, Richardson had laid the foundation of Thermionics—a subject of the greatest theoretical and practical importance. He was the first to study in detail the escape of electrons from hot bodies in a vacuum and to give the correct interpretation of the phenomena. His investigations, continued over many years, led him to originate many of the fundamental ideas connected with the emission of electrons from hot bodies which are now universally accepted. His work on photo-electric emission was also of fundamental importance, and in it many of the now generally accepted ideas relating to interaction between radiation and matter were suggested. Among many important contributions in other fields was the prediction and calculation of the gyro-magnetic effect—the rotational torque accompany the



magnetisation of a rod. During the last ten years he has continued to make many important contributions towards the clearing up of difficulties in the subjects of his earlier investigations. In addition, he has done important work on electron emission associated with chemical action. He and his students have contributed largely towards filling up the gap between the ultra-violet and X-ray spectra. His main work in recent years has related to the hydrogen molecule, and has afforded a detailed test of the new quantum mechanics when applied to one of the simplest structures for which the old quantum mechanics breaks down. Richardson has throughout his work shown a most unusual combination of experimental skill and ingenuity with theoretical knowledge and insight.

A Royal Medal is awarded to Professor John Edward Marr, F.R.S.

At a time when few believed it possible Professor Marr discerned a delicate time-scale in the Lower Palaeozoic Rocks, chiefly in the Lake District and North Wales, and applied it to elucidating the development of life and earth-structure. After testing his results in Scandinavia and in Bohemia he was able to make further use of them in setting in order corresponding rocks in South Wales. He has worked out the structure, origin and development of the mountains, lakes and rivers in Lakeland and elsewhere in the North of England, pioneer work which has been eagerly followed up by his pupils and successors. His work in association with Dr. Harker on the metamorphism brought about by the great mass of granite of Shap Fell on the rocks into which it was injected has become classic, and has inspired the rapid advance now being made in kindred studies. Of recent years he has contributed largely to knowledge of the Cambridge district, and particularly of the Pleistocene Deposits and their relation to Early Man there and in East Anglia generally. For some thirty years he gave invaluable assistance to Professor Hughes at Cambridge before succeeding him in the Chair. His labours during over forty-five years have been the chief instrument in establishing and maintaining the position in training and research now held by the Cambridge School of Geology.

The Davy Medal is awarded to Professor Robert Robinson, F.R.S.

By his investigations of the chemistry of the alkaloids, he has made notable additions to the knowledge of the structure of these complex substances, and by experiment extended by theoretical discussion he has strikingly indicated possible mechanisms of their formation in nature. His brilliant synthetic

work in connection with the colouring matters of flowers has greatly promoted the study of a group of substances of outstanding interest. His theoretical studies of the mechanism of organic reactions, in particular substitution in aromatic compounds, have led to results of great value in that they enable a very wide range of reactions to be considered from a common point of view.

The Darwin Medal is awarded to Professor Johannes Schmidt.

The Darwin Medal is given in reward of work of acknowledged distinction in the field in which Charles Darwin himself laboured. When it is remembered that the store of knowledge which Darwin accumulated on board the "Beagle" in her voyage round the world was the foundation of all his later thought and of his outlook on biological problems, the conditions of the award are without doubt fulfilled in the person of Dr. Johannes Schmidt, at the same time a distinguished oceanographer, and a recognised research worker in genetics of animals and plants. The number and extent of the voyages in small research vessels which Dr. Schmidt has accomplished with success, his large and varied collections of the pelagic fauna and flora, and the remarkable series of observations, made under his direction, on the physical and chemical phenomena of the sea, give him an undisputed place in the first rank of those scientific explorers whose labours have built up our knowledge of the oceans of the world. His researches on the life-history of the freshwater eel and the discovery of its breeding places far out in the Atlantic have proved of such general interest that they are almost as well known and appreciated by the general public as they are accepted and valued by his fellow-biologists, although in reality these researches constitute a comparatively small part of his real contributions to the science of the sea. It has already been said that, in addition to his work of marine exploration, Dr. Schmidt has devoted great attention to the fundamental problems of the science of genetics. His breeding experiments on the tropical freshwater fish, *Lebistes*, carried out in the Carlsberg Physiological Laboratory at Copenhagen, of which he is a director, are of much interest, whilst his investigations on the local races of the viviparous blenny (*Zoarces viviparus* L.) are of outstanding importance and originality.

The Hughes Medal is awarded to Sir Venkata Raman, F.R.S.

Sir Venkata Raman is one of the leading authorities on optics, in particular on the phenomenon of the scattering of light. In this connection, about three years ago he discovered that the light's colour could be changed by scattering.

This had been predicted theoretically some time before, but in spite of search the change had not been found. The "Raman Effect" must rank among the best three or four discoveries in experimental physics of the last decade. It has proved, and will prove, an instrument of great power in the study of the theory of solids. In addition to important contributions in many fields of knowledge, he has developed an active school of research in Physical Science in the University of Calcutta.

612 . III . II

*The Isolation by Cataphoresis of Two Different Oxyhæmoglobins  
from the Blood of some Animals.*

By ALEXANDER GEIGER, M.D., from the Department of Hygiene and  
Bacteriology, The Hebrew University, Jerusalem.

(Communicated by Dr. H. H. Dale, Sec.R.S.—Received September 18, 1930.)

Many observers have found specific differences in the properties of hæmoglobins from different species (Barcroft, 1928). These differences are due—as Barcroft states—to the globins, whereas the hematin is probably the same in all hæmoglobins (Barcroft, 1928).

Another important question was raised by Bohr (1892). This author advanced the hypothesis that more than one hæmoglobin may be present in the same animal. Bohr's hypothesis was also supported by Hoppe-Seyler's investigations (Hoppe-Seyler). Crystallographic observations on hæmoglobins by Reichert and Brown (1909) showed that it is possible to obtain different crystals of oxyhæmoglobin from the same blood. The oxyhæmoglobin crystals are dimorphous or polymorphous in many species of animals. A. K. Boor, criticising the findings of Reichert and Brown, states (Boor, 1930) that the conditions of crystallisation were not uniform. According to Boor, various organic impurities might have influenced the crystal habit, as well as the crystal system and form. Boor prepared oxyhæmoglobin by mixing washed red corpuscles with aluminium cream, followed by filtration in a refrigerator. The crystals were formed from the pure filtrate by adding a suitable amount of ethyl alcohol and maintaining a temperature of 0° C. or lower. Only one kind of crystal of horse oxyhæmoglobin and of horse carbon monoxide hæmoglobin is reported, while dog oxyhæmoglobin was found to be represented

by two forms, though only one form of dog carbon monoxide hæmoglobin was observed. According to Boor, every case of dual form is doubtful, and he suggests that there is only one hæmoglobin for each species.

W. Kuester (1925) claimed, on the basis of purely chemical investigations, that two hæmoglobins are present in ox blood. To quote Kuester: ". . . das Hæmoglobin wird als dreibasische komplexe Säure aufgefasst, die das Eisen im Anion fñhrt. Zwei der abdissoziierten Wasserstoffatome stammen von zwei Carboxylen des Globins, ein Wasserstoffatom von einem Carboxyl der prosthetischen Gruppe. Die beiden Carboxyle der prosthetischen Gruppe sind verschieden. Es müssen also zwei Hæmoglobine existieren. Im Hæmoglobin Aa ist das starke Carboxyl *a* mit der prosthetischen Gruppe verknuepft, im Hæmoglobin Ab das schwache. . . ."

The failure to isolate, hitherto, two or more oxyhæmoglobins (or hæmoglobins) from the same blood with any degree of constancy constitutes the main obstacle to a definite conclusion as to the existence of two or more hæmoglobins in one animal. In this paper it is shown that it is possible to isolate two oxyhæmoglobins from the blood of an ox, dog, sheep or ass. Two oxyhæmoglobins were also found in a few samples of human blood.

#### *First Method of Separation.*

The following investigations were carried out with cataphoresis:—

Two proteins existing in a common solution can be separated by cataphoresis, if one of them bears a positive and the other a negative charge. Owing to their ampholytic character the nature of the charge on proteins (which decides the direction of migration in the electric field) depends largely on the hydrogen-ion concentration of the solution. The  $p_H$  effecting the change of the electric charge of a protein from positive to negative, or *vice versa*, i.e., the isoelectric point, represents a constant, depending on the character of the protein and the composition of the solvent medium. When the hydrogen-ion concentration of the solvent containing two proteins lies between the isoelectric points of the latter, the electric charges on these proteins are of opposite signs. Further, if such a solution is subjected to cataphoresis, the two proteins migrate in opposite directions.

Such a method of separation can, of course, be employed only when the distance between the isoelectric points is sufficiently large to enable both proteins to show a distinct migration under the above described conditions. The cataphoresis of oxyhæmoglobin has been repeatedly examined (Michaelis und Davidsohn, 1912) without detection of bipolar migration. This may be

explained by the fact that the isoelectric points of both hæmoglobins existing in a particular blood are near to each other. In these circumstances it is possible for the conditions of the experiment to prevent the occurrence of a sharp isoelectric point, a large isoelectric zone being formed which obscures the small differences existing between the two individual isoelectric points. The sharpness of the isoelectric point depends on the composition of the solvent medium. The higher the electrolyte concentration of the solvent medium, the less sharp the isoelectric point; in other words, the wider the  $p_H$  zone within which protein does not show cataphoretic migration. The  $p_H$  of the isoelectric point is also influenced by the chemical character of the electrolytes present.

Preliminary observations, to be published separately, showed that phosphate ions have a strong electrical discharging effect upon hæmoglobins. In a phosphate buffer solution of 1/15 mols., hæmoglobin shows a wide isoelectric zone, ranging from 0.3 to 0.4  $p_H$ . As most workers have used comparatively strong phosphate concentrations in their investigations, it will be readily understood why they were unable to obtain a separation of the two hæmoglobins. The isolation of both hæmoglobins from blood was only possible after the conditions influencing the sharpness of the isoelectric point and its position in the  $p_H$  scale had been determined. It was found by preliminary experiments that the isoelectric point of hæmoglobins from ox blood occurs at  $p_H$  6.7 to 6.9 in a mixture of  $m/15$   $Na_2HPO_4$  and  $m/15$   $KH_2PO_4$ .

In the first place an attempt was made to obtain separation by lowering the phosphate concentration, and this was successful. But under these conditions the separation proceeded so slowly, in an ordinary cataphoresis apparatus, that a change of the composition of the solution under examination was to be feared. A special apparatus was therefore constructed. Every experiment was carried out with the apparatus of Michaelis and Dombovicianu (1924) and also with our own apparatus.

In these experiments undialysed hæmoglobin was used, but as will be shown later, a considerable improvement in the separation can be obtained by using electrodialysed hæmoglobin.

The hæmoglobin was prepared in two different ways: (1) The erythrocytes, which were washed four times with 1 per cent. NaCl solution, were hæmolyzed with water. The stromata were removed as far as possible by prolonged centrifuging. (2) Well-washed erythrocytes were shaken with toluol and kept overnight in an ice chest. With this treatment it was possible to separate the stromata from hæmoglobin by centrifuging.

The solution actually used for examination contained phosphate buffers in a concentration of 0.02 mol., and the concentration of hæmoglobin was from 1 to 2 per cent. A series of such solutions with different  $p_H$  values was prepared, the  $p_H$  interval between successive solutions being approximately 0.03. The solution contained in the side-cells of the cataphoresis apparatus had, in every experiment, the same  $p_H$  and phosphate concentration as that in the central cell.

The electrometric measurements of the  $p_H$  of these solutions was carried out before and after cataphoresis, with a somewhat modified Clark's hydrogen electrode. Only those experiments were recorded in which the solutions employed showed no marked change of  $p_H$  during the time of experiment.

These experiments definitely showed that, at a properly chosen  $p_H$ , migration of hæmoglobin towards both poles was taking place simultaneously. Though under these conditions migration proceeds very slowly, its progress towards both poles is steady. The migration velocity amounted to 3 to 4 mm. per hour towards both poles, at a properly chosen  $p_H$  in the apparatus employed, with a potential difference of 230 volts at the poles. It was observed that when stroma substances were left behind in the solutions examined, they migrated to the positive pole, a sharply defined turbid layer being observed. The hæmoglobin layer always showed a distinctly visible separating surface.

The same bipolar migration was observed when the cataphoresis apparatus of Michaelis was used, the presence of two differently charged hæmoglobins being thus indicated.

In the case of ox blood the  $p_H$  required to effect separation showed small individual variations, which may be explained by the presence of substances, and notably of electrolytes, in the solutions examined. Hæmoglobins derived from over 20 samples of ox blood and examined by the method above described always gave the same result. Separation of the two hæmoglobins was, therefore, always reproducible, provided that the conditions detailed above were adhered to, and the  $p_H$  carefully adjusted.

The same experiments were carried out with the blood of dogs, sheep and asses. They all showed the same phenomenon of bipolar migration, demonstrating the existence of two different hæmoglobins, although the  $p_H$  required to effect separation was found to vary with the animal species.

In the case of human blood, however, this method did not yield conclusive results. Of five samples examined only one yielded distinct separation with the above method.

In Table I the  $p_H$  values at which simultaneous migration to both poles was

observed are recorded. Only a few of the concordant results are reported here.

Table I.

EXPERIMENTS WITH OX BLOOD.		EXPERIMENTS WITH DONKEY BLOOD.	
Sample No. 1	$p_H$ 6.81-7.04	Sample No. 1	$p_H$ 6.62-6.78
3	6.86-7.05		6.67-6.76
4	6.96-7.06		
5	6.81-7.00		
6	6.76-6.82		
7	6.74-6.80		
8	6.87-6.94		
9	6.80-6.98		
10	6.77-6.86		
	6.82-6.88		
EXPERIMENTS WITH DOG BLOOD.		EXPERIMENTS WITH SHEEP BLOOD.	
Sample No. 1	$p_H$ 6.47-6.81	Sample No. 1	$p_H$ 6.77-6.83
2	6.47-6.72	2	6.58-6.80
3	6.48-6.85	3	6.53-6.80
4	6.55-6.83	4	6.62
5	6.50-6.91	5	6.65

An important point in this investigation was to determine whether cataphoretically separated hæmoglobin will show unipolar migration, when re-subjected to cataphoresis at the same  $p_H$  at which separation of hæmoglobins was originally effected. This point was examined in a series of experiments. It was actually found that hæmoglobin originally separated at the anode, migrated to the anode, and exclusively so, at the  $p_H$  at which the separation originally took place, whereas the other hæmoglobin migrated exclusively to the cathode under the same conditions.

This second cataphoresis experiment has been made also at different hydrogen concentrations of the solutions. In these cataphoresis experiments with separated hæmoglobins only unipolar migration was observed. Thus convincing proof is afforded of the actual existence of two different hæmoglobins in the samples examined. The results of these experiments are given in Table II (p. 373).

#### *The Improved Method of Separation with Electrodialysed Hæmoglobin.*

Though the results above described proved conclusively the co-existence of two hæmoglobins, the yield obtained was so small as to preclude detailed examination. The assumption has been previously made that hæmoglobins

Table II.

Original solution.	Haemoglobin separated at the anode and resubjected to cataphoresis.		Haemoglobin separated at the cathode and resubjected to cataphoresis.		Remarks.
	At $p_H$ .	Migration.	At $p_H$ .	Migration.	
Ox haemoglobin, separation at $p_H$ 6.88 in 0.02 mol. phosphate buffers	6.72	To cathode	6.87	To cathode	0.02 mol. phosphate buffers in the side cells.
	6.76	None	6.90	"	
	6.80	To anode	6.94	"	
	6.87	"	6.98	To anode	
Ox haemoglobin, separation at $p_H$ 6.77 in 0.02 mol. phosphate buffers	6.71	To cathode	6.77	To cathode	0.02 mol. phosphate buffers in the side cells.
	6.74	"	6.81	"	
	6.76	To anode	6.88	"	
	6.80	"	6.97	To anode	
Ox haemoglobin, electro-dialysed, separated at $p_H$ 7.14	6.97	To cathode	7.14	To cathode	0.005 n. NaCl in the side cells.
	7.02	None	7.17	"	
	7.07	To anode	7.21	None	
	7.14	"	7.24	To anode	
Ox haemoglobin, electro-dialysed, separated at $p_H$ 7.15	6.92	To cathode	7.15	To cathode	0.005 n. NaCl. in the side cells.
	6.99	"	7.19	"	
	7.03	None	7.22	None	
	7.08	To anode	7.25	To anode	
Ox haemoglobin, electro-dialysed, separated at $p_H$ 7.14	6.98	To cathode	7.15	To cathode	0.005 n. NaCl in the side cells.
	7.03	None	7.24	To anode	
	7.09	To anode			
	7.14	"			
Dog haemoglobin, electro-dialysed, separated at $p_H$ 6.62	6.62	To anode	6.63	To cathode	0.02 mol. phosphate buffers.
Dog haemoglobin, separated at $p_H$ 6.64	6.64	To anode	6.64	To cathode	0.02 mol. phosphate buffers in the side cells.
Human haemoglobin, electro-dialysed, separated at $p_H$ 6.90	6.90	To anode	6.90	To cathode	0.005 n. NaCl in the side cells.
Human haemoglobin, electro-dialysed, separated at $p_H$ 6.88	6.89	To anode	6.88	To cathode	0.005 n. NaCl in the side cells.

are discharged by electrolytes. The electrolyte contents of the haemoglobin solutions were consequently reduced to a minimum by employing electro-dialysis.

The haemoglobin solution prepared by the above-mentioned toluol method was electro-dialysed until its conductivity showed no further appreciable change.



The apparatus employed resembled that of Stadie and Ross (1926). The side parts of the apparatus were made of glass. The middle cell was cut out of a thick rubber plate. Two plates of carbon were employed as electrodes. In most experiments a membrane-combination according to Ettisch and Ewig (1929) was adopted, *i.e.*, a collodion membrane previously immersed in a gelatine solution was used at the anode and parchment paper at the cathode. The maximum initial intensity of the current was 60 milliamperes. The temperature during dialysis was kept below 25° C. These conditions were easily controlled by using the resistance of a 15-watt lamp and by frequently changing the water in the apparatus. The distance between the membranes was about 1 cm. and the surface area of each membrane was approximately 50 cm.<sup>2</sup>. Continuous stirring of the hæmoglobin during electro-dialysis is required, because methæmoglobin and some hæmoglobin precipitates on the anodic membrane if stirring is omitted. With adequate stirring, a pure, concentrated oxyhæmoglobin solution is obtained, which contains no stroma substances. The stroma substances remaining in the hæmoglobin solution after treatment with toluol are precipitated during electro-dialysis.

In order to observe the course of electro-dialysis samples were withdrawn from the hæmoglobin solution at short intervals. The total base content, the Cl concentration and the  $p_H$  of these samples were determined. The analysis of the total base content was carried out by the method of Stadie and Ross (1925): the concentration of Cl was determined by Wilson's method (Wilson and Ball, 1928), and hydrogen-ion concentrations were measured electrometrically in a Clark electrode slightly modified for our purpose.

Table III shows the typical course of the electro-dialysis of ox hæmoglobin.

From Table III it is evident that the electro-dialysis is a harmless process, if properly conducted. It was usually continued until the hæmoglobin solution

Table III.

Times at which sample was withdrawn.	$p_H$ .	Concentration of Cl expressed in milli-eq. per litre.	Total base concentration expressed in milli-eq. per litre.	Temperature.
Beginning .....	7.49	93.2	161.9	° C. 16
After 45 minutes .....	7.38	40.0	90.8	19
After 90 minutes .....	7.35	3.0	30.0	22
After 125 minutes .....	7.21	0	5.5	23
After 300 minutes .....	7.04	0	0.4	18

reached  $p_H$  7.15. After removing the impurities by the centrifuge, this solution was submitted to cataphoresis.

The cataphoresis was carried out as follows. The middle cells of the apparatus were filled with the concentrated, electrodialysed hæmoglobin solution, a NaCl solution of about the same conductivity being placed in the side cells. The connection between the apparatus and the electrodes was established by agar bridges, when an apparatus of the type devised by Michaelis and Dombovicianu was used. By this method ox, sheep, and human hæmoglobins have been examined. Two hæmoglobins could be easily isolated from each sample of ox and sheep blood. The optimum hydrogen-ion concentration for the separation is  $p_H$  7.15 for ox blood, and 7.05 for sheep blood. The separation of the two hæmoglobins is not influenced by the concentration of hæmoglobin, but it is influenced by that of electrolytes. With this method enough material can be obtained for further studies of both hæmoglobins. Under the conditions described the migration of the hæmoglobins towards both poles proceeds quickly and steadily, so that the cataphoresis apparatus of Michaelis and Dombovicianu can be used without fear of changes in the H-ion concentration. The migration velocity of the ox hæmoglobins amounts on an average to 19 to 20 mm. per hour toward both poles at  $p_H$  7.15, using a 20 per cent. hæmoglobin solution and an apparatus of the Michaelis type, in which the decline of the potential amounts to 2.5 volts per centimetre, when the conductivity of the solution contained in the middle cell has the same value as that in the side cells. Under identical conditions the hæmoglobins, from a 24 per cent. solution of sheep hæmoglobin migrated with an average velocity of 8.5 to 11 cm. per hour toward both poles at  $p_H$  7.05. Table IV shows some typical results. From every sample examined, without exception, both hæmoglobins could be separated.

In Table IV a few examples only are given from numerous experiments made on ox blood.

The separated hæmoglobin solutions were in most cases subjected to cataphoresis a second time, the result being unipolar migration exclusively. I propose to call the one hæmoglobin, migrating to the anode during separation, *hæmoglobin A*, and the other, migrating at the same time to the cathode, *hæmoglobin B*.

Human hæmoglobin does not behave like ox or sheep hæmoglobin. In the few undialysed samples examined, it was not always possible to obtain separation, using phosphate buffers. From electrodialysed human hæmoglobin two hæmoglobins can be separated by very carefully adjusting the  $p_H$ . Owing

Table IV.

Sample.	$p_H$ of original hæmoglobin solution.		Migration velocity to the anode, cm.-hour.	Migration velocity to the cathode, cm.-hour.	$p_H$ of solution migrating to anode.	$p_H$ of solution migrating to cathode.	Decline of potential, volts per cm.
	Before cata- phoresis.	After cata- phoresis.					
Ox hæmoglobin, 23 per cent.	7.13	7.12	14	27	7.12	7.12	2.5
Ox hæmoglobin, 20 per cent.	7.15	7.15	19	21	7.15	7.15	2.5
Ox hæmoglobin, 25 per cent.	7.14	7.15	18	17	7.14	7.15	2.5
Ox hæmoglobin, 24.5 per cent.	7.13	7.13	13	22	7.13	7.12	2.5
Sheep hæmoglobin, 24 per cent.	7.05	7.05	8	8	7.04	7.05	2.5
Sheep hæmoglobin, 18 per cent.	7.03	7.04	6	8	7.04	7.04	2.5
Sheep hæmoglobin, 12 per cent.	7.05	7.04	7	11	7.04	7.04	2.5
Human hæmoglobin, 4 per cent.	6.89	6.90	4	6	6.90	6.90	2.5
Human hæmoglobin, 13 per cent.	6.88	6.88	5	5	6.89	6.88	2.5

to the small number of observations made on human hæmoglobin, further studies are required.

Ox hæmoglobins were separately studied. To obtain larger quantities of both hæmoglobins, a large cataphoresis apparatus was used, devised by Coehn (1909), and consisting of a large U-tube of about 20 mm. inner diameter, provided with two large stop-cocks at the lower end. The upper ends of the side parts of the apparatus were connected together by another smaller stop-cock. In both upper ends a thinner, doubly recurved glass tube, filled with a 3 per cent. agar solution containing 10 per cent. NaCl, was inserted. The upper part of the curvature of this tube is filled with the same solution as that contained in the side parts of the cataphoresis apparatus. This tube connected the apparatus with the electrodes, consisting of copper wires immersed in a copper sulphate solution. These tubes were sufficient to prevent diffusion of copper sulphate or NaCl into the apparatus during a cataphoresis lasting about 12 hours. During this time about 15 c.c. of both hæmoglobins could be obtained on the two sides of the apparatus.

It is to be observed that the solution of the migrating hæmoglobin becomes diluted when the bores of the stop-cocks are smaller than the diameter of the

apparatus. Dilution also occurs when the conductivity of the NaCl solution contained in the side parts has a lower value than that of the hæmoglobin solution. To prevent bacterial decomposition during cataphoresis it is advisable to place the whole apparatus in an ice chest. By this method larger quantities of the two hæmoglobins have been separated, and the oxygen dissociation curves of both have been examined.

*Oxygen Dissociation Curves.*

As these experiments, dealing with the oxygen dissociation curves of the two hæmoglobins under various conditions, are not yet completed, they will be published later. I record here only one out of a number of experiments.

The curves shown in fig. 1 were drawn from measurements made on ox hæmoglobin solutions, separated from electrodialysed solution. Both had 10 milli-mols. NaCl per litre, the  $p_H$  of both being 7.14. An electrodialysed hæmoglobin solution derived from ox blood was submitted to cataphoresis at  $p_H$  7.14. At the end of the cataphoresis the hæmoglobin solutions, which had migrated simultaneously to both poles of the apparatus, were withdrawn, and their oxygen dissociation curves were separately determined. The saturation was made at 26° C. in Barcroft tonometers. The oxygen percentage in the saturators was determined with a Haldane apparatus, and the oxygen contents of the blood samples were analysed by Van Slyke and Neill's manometric method (1924).

As fig. 1 shows, at 10 mm.  $O_2$  tension hæmoglobin A is saturated only to

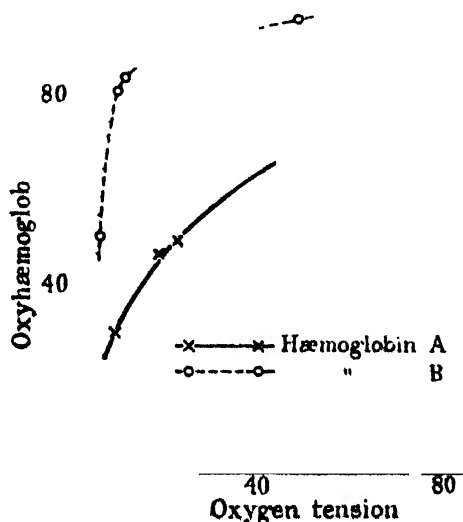


FIG. 1.

45 per cent. with oxygen, while at the same oxygen tension hæmoglobin B consists of 85 per cent. oxyhæmoglobin. This experiment clearly demonstrated the differences existing between two hæmoglobins separated from the same blood and amply confirms the evidence given by cataphoresis of the existence of two hæmoglobins in ox blood.

#### *Discussion of the Results.*

The existence of the two different hæmoglobins in an individual blood has been demonstrated mainly by the simultaneous dipolar migration of hæmoglobins. As the bipolar migration of electrodialysed hæmoglobin solution takes place at a  $p_H$  which was hitherto assumed to be the "isoelectric point" of hæmoglobin, it has to be considered whether an isoelectric solution of an individual protein could show bipolar migration. The following review will show that this cannot be the case.

As the direction of migration of a protein depends on the nature of its electric charge, in spite of its ampholytic character, it can be positive or negative, and at the  $p_H$  of its "isoelectric point" no migration at all will take place. The assumption that such a body could have a surplus of positive and negative charge at the same time would be absurd, and therefore such an explanation of the observed phenomenon of bipolar migration is impossible.

It remains to be considered whether the experimental conditions of cataphoresis described above would allow a simultaneous bipolar migration of a *single* protein to occur.

Linder and Picton (1927) observed a reversal of the movement of proteins in the course of a cataphoresis experiment, the proteins examined changing their original direction of migration after longer exposure to electric current. This phenomenon has been explained by Hardy (1899), who showed that it is caused by change of the charge of the protein, due to products of electrolysis. The technique used by Linder and Picton did not provide for depolarisation or prevent the products of electrolysis from entering the cataphoresis cell.

In our experiments, using unpolarisable electrodes, and connecting the apparatus by means of long agar-filled tubes with the electrodes, no products of electrolysis could enter the apparatus. In the first experimental series in which diluted hæmoglobin solutions were subjected to cataphoresis, buffering with phosphates prevented slight changes of  $p_H$ , while in the second experimental series, using electrodialysed hæmoglobin solutions, the high concentration of hæmoglobin provided a sufficient buffering effect. By measuring the

H-ion concentrations before and after cataphoresis, changes in the H-ion concentration, as a cause of bipolar migration, have been excluded.

In Linder and Picton's experiment the protein changed its original direction of migration, while in our experiments simultaneous migration toward both poles took place, and, changes in *pH* being excluded, no change of the original charge was possible. It is assumed, therefore, that two hæmoglobins, bearing opposite electric charges, were present in the solution. This assumption is also confirmed by the fact, that if a sample of separated hæmoglobin was submitted to cataphoresis a second time, unipolar migration was always observed, at every *pH*.

Interference may also be caused owing to irregular heating of the apparatus by the electric current, because the hæmoglobin solution rises irregularly on the heated parts. This factor was excluded in the second experimental series with electrodialysed hæmoglobin solution, because of the low conductivity of the solutions. By using adequate resistances the heating of the apparatus was prevented in the first series of experiments. The effect of electroendosmosis of water on the migration of colloids can be observed in a microscopical cataphoresis experiment. This effect, which shows itself in the opposite movement of the particles nearest to the glass walls, has no significance with the macro-cataphoresis method when the internal diameter of the apparatus is sufficiently large. As Kruyt (1928) states, only the sharpness of the migrating surface can be influenced by electroendosmosis in the macroscopical method. We always observed sharp migrating surfaces toward both poles.

It is to be noted that differences between the conductivity of the solution examined and that contained in the side parts of the apparatus influences the migration velocity of the colloid and the concentration of the migrating sol, but can never change the direction of the migrating particles.

It is certain that no simultaneous, bipolar migration of a single protein can occur. The hæmoglobin solution examined contained positive as well as negative particles, at the *pH* at which bipolar migration was observed. As the same body cannot bear simultaneously a surplus of positive and negative charge, it is evident that two different hæmoglobins were present. This has been experimentally proved by the fact that the hæmoglobin, when once separated, did not again show bipolar migration. The H-ion concentrations corresponding to the isoelectric points of the two hæmoglobins have different values.

A further evidence of the real difference between the two hæmoglobins separated by cataphoresis is furnished by their different oxygen dissociation curves.

*Summary.*

1. The factors influencing the magnitude and the nature of electric charge of proteins are discussed.
2. Two different hæmoglobins have been separated from ox, sheep, ass, dog and human blood. It was not possible to isolate two hæmoglobins from every sample of undialysed human blood when phosphate buffers were used.
3. It is easier to isolate the two hæmoglobins from a given blood if the hæmoglobin solution is electrodialysed prior to cataphoresis. By this method two hæmoglobins can be obtained also from human blood.
4. The oxygen dissociation curves of the two hæmoglobins isolated by cataphoresis have been determined and found to be different.
5. Possible sources of error have been discussed and excluded.

## REFERENCES.

- Barcroft, J. (1928). "The respiratory function of the blood: II. Hæmoglobin." Cambridge, pp. 38-51.
- Barcroft, J. (1928). 'Bull. Soc. Chim. Biol.,' vol. 10, p. 279.
- Bohr, Chr. (1892). 'Skand. Arch. Physiol.,' vol. 3, p. 76.
- Hoppe-Seyler (cited by Hammarsten). 'Lehrbuch Physiologischen Chem.'
- Reichert and Brown (1909). 'The Crystallography of Hæmoglobins.' Washington.
- Boor A. K. (1930). 'J. Gen. Physiol.,' vol. 13, p. 307.
- Kuoster, W. (1925). 'Z. Physiol. Chem.,' vol. 151, p. 56.
- Michaelis und Davidsohn (1912). 'Biochem. Z.,' vol. 41, p. 102.
- Michaelis und Airila (1921). 'Biochem. Z.,' vol. 118, p. 144.
- Michaelis und Dombovicianu (1924). 'Koll. Z.,' vol. 34, p. 322.
- Stadie and Ross (1926). 'J. Biol. Chem.,' vol. 68, p. 229.
- Ettisch und Ewig (1929). 'Biochem. Z.,' vol. 216, p. 430.
- Stadie and Ross (1925). 'J. Biol. Chem.,' vol. 65, p. 735.
- Wilson and Ball (1928). 'J. Biol. Chem.,' vol. 79, p. 221.
- Coehn (1909). 'Z. Elektrochem.,' vol. 15, p. 653.
- Van Slyke and Neill (1924). 'J. Biol. Chem.,' vol. 61, p. 523.
- Linder and Picton (1897). 'J. Chem. Soc.,' vol. 71, p. 568.
- Hardy (1899). 'J. Physiol.,' vol. 24, p. 292.
- Krulyt. 'Koll. Z.,' vol. 37, p. 358.
- Krulyt and Van der Willigen (1928). 'Koll. Z.,' vol. 44, p. 22.

*The Origin of the Mesoderm in the Gastropod Viviparus*  
(= *Paludina*).

By WILFRED FERNANDO, B.Sc. (Lond.), Ceylon Government University Scholar, Marshall Scholar, Imperial College of Science and Technology.

(Communicated by E. W. MacBride, F.R.S.—Received October 8, 1930.)

[PLATES 23–28.]

*Introduction.*

For the first account on the development of the mesoderm in *Viviparus viviparus* (L.) [= *Paludina vivipara* Férussac] we are indebted to Bütschli (1877). According to him the mesoderm arises as in other Gastropoda from two mesoblastic bands. At first each of these bands consists of only a few cells, but later on the number of cells increases, followed by the breaking up of the two bands and subsequent distribution of the mesoderm cells in the cleavage cavity.

In 1891 Erlanger's (1891) exhaustive description of the embryology of *Viviparus* appeared, in which he made the startling discovery that the coelom arises as a pocket from the archenteron. This sac is the "primary coelom"; it becomes bilobed and its connection is severed with the archenteron. Later on the cells lining it break up and spread irregularly through the segmentation cavity. From the mesenchymatous cells thus produced is developed the pericardium, which represents the "secondary coelom." Thus Erlanger was able to bring the Mollusca into line with the Annelida and the Echinodermata with respect to the early developmental stages.

While Erlanger's results were accepted by many workers, others remained unconvinced of the accuracy of his observations. Korschelt (1893) in his description of the development of mesoderm in Mollusca in the 'Text-book of the Embryology of Invertebrates,' says: "Considering all that is as yet known of the formation of the mesoderm we cannot agree with the results obtained by Erlanger for *Paludina*, and must continue to be sceptical about them until they are better supported or are actually confirmed by new investigations (if possible on other forms as well)."

In reply to this Erlanger (1894) described again the development of the



mesoderm which he supplemented with photomicrographs of his sections. In one series he found in the wall of the hinder part of the gut on each side a large endoderm cell which was actively dividing. From these two cells he believes that the coelomic sac is formed and he homologises them with the pole-cells of other Mollusca.

In 1896 Tönniges (1886) repeated Erlanger's work. He disagreed with the descriptions of both Bütschli and Erlanger. According to Tönniges the mesoderm arises as a result of the migration of cells from the ventral ectoderm layer, near the blastopore, into the segmentation cavity. Tönniges, however, made no reference to Erlanger's later work of 1894.

In 1905 a further contribution to the embryology of *Viviparus* was made by Otto and Tönniges (1905), and again Tönniges found no confirmation of Erlanger's description of the enterocelic method of mesoderm development, but, on the contrary, confirmed his own earlier conclusions.

No further attempts were made to verify the contradictory statements made by Erlanger and Tönniges till 1929, when Dautert (1929) described the formation of the germ layers in *Viviparus*. He also found no support for Erlanger's views, but according to him, "Das Mesoderm der *Paludina* entsteht durch auswanderung von Zellen der vorderen Ectodermwand unterhalb des Velums."

The present investigation is an attempt to settle by confirmation or otherwise of Erlanger's work on *Viviparus* the problem presented by the conflict of views outlined above. It was carried out at the Zoological Department of the Imperial College of Science and Technology at the suggestion of Prof. E. W. MacBride, F.R.S., to whom I am under many obligations for his advice and encouragement which made this research possible. I am also indebted to Mr. H. R. Hewer and Mr. T. L. Green for helpful suggestions and criticisms.

### *Technique.*

*Viviparus* is viviparous and development takes place in the enlarged oviduct which is termed the uterus. The embryos were washed out of the uterus into salt solution, and were fixed in Kleinenberg's picro-sulphuric acid, Perenyi's chromo-nitric acid and Flemming's solution. They were embedded in celloidin and paraffin and stained in Delafield's hæmatoxylin.

Kleinenberg's fixative gave results which were inferior to those yielded by the other two. Fixation in Kleinenberg produces a greater amount of vacuolisation and a general loosening of the cells. The result of this is that it is very difficult to differentiate the mesoderm cells from the rest of the ectoderm and endoderm. Flemming's solution gives the most satisfactory results, because

there is no loosening of cells and the various cavities in the embryo appear quite distinct. Sections of material fixed in Flemming's solution were left in borax carmine, as recommended by MacBride (1915), before transference to hæmatoxylin.

Dautert is inclined to believe that Erlanger's results were due to faulty preparation. In this connection I have to remark that Erlanger used as fixative, in addition to Flemming, Kleinenberg's picro-sulphuric acid to which he added a drop of osmic acid, whereas Dautert employed Kleinenberg's fixative without osmic. Further I find that Kleinenberg is quite inferior to Flemming's chromo-osmic. In fact, if all the material had been fixed in Kleinenberg alone there is little doubt that the result of the present investigation would have been to confirm Dautert's views.

#### *Description.*

The segmentation of the egg of *Paludina* is described by Dautert (1929). The egg is minute and contains very little yolk. Segmentation is total and results in a blastula of almost equal-sized macromeres and micromeres. By an invagination of cells into the segmentation cavity a gastrula is produced, which has a wide archenteron and a restricted segmentation cavity. At the anterior end of the embryo the velar cells are differentiated in the ectoderm and cilia are developed on them. This results in the embryo attaining the trochophore stage. The mesoderm begins to develop at this stage.

In order that the early development of the mesoderm may be clearly understood, a series of sections of the very early trochophore cut sagittally will be described.

Fig. 1 (Plate 23) is a section which passes through the side of the blastopore and archenteron. The dorsal surface of the embryo is almost flat and the ventral surface is round. At the posterior end, the ectoderm is seen to surround the opening of the blastopore which is represented by a small space. The archenteric wall appears as a solid mass of cells with a cavity at the anterior ventral side which is a part of the archenteron. The cells of the ectoderm are characterised by greater vacuolisation and at the anterior end the larger velar cells are found. The endoderm cells at the anterior end are more vacuolated than those nearer the blastopore.

On examining the segmentation cavity at the anterior end we find lying in it a distinct cavity lined by small cells which in appearance approach more closely the endoderm cells at the posterior end of the embryo. This cavity lies almost ventral to the archenteron and is seen to be continued posteriorly

towards the blastopore, as the same type of cells which surrounded the cavity are also found here. This is the lateral portion of the coelom.

The next section (fig. 2) passes through the blastopore. In outline this section resembles the previous one. The vacuolisation of the anterior endoderm cells is more distinct than in the previous section. The segmentation cavity above the archenteron is smaller than the ventral portion.

On the ventral wall of the archenteron near the blastopore there is a distinct sac. This sac lies almost at the commencement of the archenteron between the outer ectoderm and the inner endoderm. The cells lining this lower cavity are small, and can be readily distinguished from those of the ectoderm. There is no evidence that they are derived from the latter. This sac is the coelom which was first described by Erlanger.

In fig. 3 we find again this coelom near the blastopore. It is now shown mid-ventral in position.

If we reconstruct from these three sections we can readily visualise a distinct median ventral coelomic cavity, which has the tendency to produce lateral lobes, growing out from the archenteron. A reconstruction of half an embryo at this stage is shown in fig. 4 (Plate 24).

The segmentation cavity is larger at the anterior end of the embryo, so that the archenteron, which now begins to grow, naturally comes to occupy this space. At the same time the archenteron presses against the coelomic sac, and as a result the latter begins to lie more closely against the ectoderm wall and the endoderm of the archenteron. The archenteron, in sagittal section, appears to be larger at the anterior end than more posteriorly, which shows that it is beginning to grow ventrally at the anterior end. As it grows thus into the segmentation cavity so as to occupy the entire space anteriorly, the forward growth of the coelom is prevented. The result of this is that the coelom now begins to grow laterally towards the dorsal surface, so that a small median coelomic cavity is found lying ventral to the archenteron and closely pressed by it, with a lateral pouch on either side growing dorsally.

This is made clear by examination of a series of frontal (horizontal) sections of an older trochophore.

Fig. 5 is a section of the dorsal part of the trochophore. The edge of the blastopore is visible posteriorly. The ectoderm cells are on the outside and archenteric cells in the middle. The segmentation cavity is not distinct but a patch of small cells on each side of the median line represent the most dorsal portion of the lateral coelomic pouches.

In fig. 6 the blastopore leads into the archenteron. The anterior wall of

the archenteron is thick and much vacuolated, and almost touches the ectoderm. The velar cells are distinct. In the segmentation cavity on each side is the coelom, a distinct cavity, enclosed by cells smaller and flatter than those of either the ectoderm or endoderm. The coelomic cavity is almost obliterated by being pressed against the ectoderm and the endoderm by growth of the archenteron. These cells forming the coelom do not contain vacuoles as do those of the ectoderm or the endoderm and are readily distinguishable from both.

In fig. 7 (Plate 25) the section passes through the ventral portion of the blastopore. At the posterior end the outer wall of the coelom is connected with archenteric cells near the blastopore. In the same way the inner coelomic wall is in connection with the wall of the archenteron. Careful examination shows that the coelom is divisible into three parts: at the posterior end is a median space which opens into the archenteron above and lies immediately below the latter and from this median cavity smaller lateral spaces grow out one on each side of the archenteron.

In fig. 8 the coelomic wall, though still retaining connection with the archenteric wall, is beginning to become separated from the latter.

In fig. 9 the coelom appears as a cavity which is lying loosely in the ventral region of the embryo. More dorsally (fig. 7) the median space was in connection with the archenteron. Further, the segmentation cavity at the posterior end is larger than at the anterior end, so that it accommodates the coelom and the median part is larger than the two lateral lobes.

A section passing more ventrally is represented in fig. 10 (Plate 26). This section passes along the ventral wall of the median coelomic cavity, so that we see only the cells lining it. The archenteron is seen to extend more towards the posterior part of the embryo than in previous sections. Still the lateral coelomic cavities are distinguishable. Hence it is evident that when the original coelomic sac was pressed against the ectoderm by the archenteron it formed two lobes on either side. Each lobe began to grow toward the dorsal surface and at the same time each grew somewhat ventrally below the ventral sac. In fig. 11 we see only parts of the walls of the ventral coelom on either side of the archenteron.

From figs. 5 to 11 the embryo as it appears when observed from its dorsal surface may be reconstructed (fig. 12). (Part of the dorsal half of the embryo is removed.)

These early developmental stages are passed through very rapidly. MacBride reports that Erlanger told him that he had considerable difficulty in obtaining

these early stages (MacBride, 1915, p. 313). This difficulty is increased by the fact that artificial fertilisation is impossible. The only chance left is to section at random a large number of embryos at the beginning of the breeding season. While young embryos were found from the end of May, the above stages were obtained only in the first few days when the breeding season had just begun. It always happened that the embryos were either in the very early segmenting stages or were past the stages of coelom formation.

As we have shown above, the coelom is in communication with the archenteron at the posterior end of the embryo, in other words the coelom arose as a hollow sac from the archenteron. The latter grows very rapidly and is always very large. Its cavity is filled with a fluid which stains very deeply with hæmatoxylin. During its growth it closes up the connection between it and the coelom. This stage is indicated in fig. 13 (Plate 27). In this section, which is a transverse section passing through the posterior region of the trochophore, the archenteron is completely cut off from the coelom below. On the dorsal surface the archenteric wall touches the ectoderm and the outlines of the cells are not quite distinct. Although the archenteron is now shut off from the coelom and communicates only with the exterior by the blastopore, the mesoderm has still not detached itself from the ventral wall of the archenteron. While the lateral lobes are lined by coelomic cells, which are mesoderm, the median cavity is lined dorsally by the archenteron and laterally and ventrally only by mesoderm.

In the segmentation cavity on one side a rudimentary cavity is seen at the top of one of the coelomic pouches. The cells lining it resemble the mesoderm cells of the coelom so that we can infer that it is budded off from the coelom. It is quite possible that if its fate be traced further it will turn out to be the larval kidney which Erlanger asserted was mesodermal in origin and budded off from the coelom. He points this out in fig. 4A of his paper in 1894. Though strongly in favour of Erlanger's view, we have not been able to follow the development of the larval kidney.

The coelom so far appears to be uniformly lined by mesoderm cells, which are readily differentiated from the ectoderm and the endoderm. With the further growth of the coelom these cells multiply rapidly and no longer form a uniform lining of the coelomic cavity. This takes place after the coelom is completely cut off from the archenteron. Fig. 14 illustrates this stage. Like the previous section it is a transverse section of the posterior region of the embryo. The coelom is a cavity which is now separate from the archenteron. The cells forming it are small and very irregular in shape. As it apposes the endoderm

on the one side and the ectoderm on the other we can thus differentiate between splanchnic and somatic layers. These mesoderm cells tend to assume an amoeboid shape and in many places they fit into the spaces between cells of the endoderm layer and ectoderm layer, and in many instances it appears as if they are budded off from the latter. As MacBride suggested, stages like these can easily lead to the view that the mesoderm originates also from the ectoderm.

This section shows two more interesting points. As we have remarked before, the coelom grows dorsally but the segmentation cavity on the dorsal region is small, so that the coelom naturally is closely pressed against the ectoderm and the endoderm. With the rapid growth of the coelom the mesoderm cells scatter freely about the dorsal surface of the archenteron and come to form a sheet of tissue between the ectoderm and the endoderm. Thus the mesoderm on the dorsal surface of the embryo is produced by the lateral coelomic sacs growing into the narrow segmentation cavity. The coelomic cavities themselves do not exist in this region, but the individual mesoderm cells produced from their walls multiply rapidly and produce the mesoderm layer.

The second noteworthy feature in this section is that the coelomic cavity appears to become larger and the mesoderm cells are seen to project into its cavity and later to break loose in it.

The somatic and splanchnic layers become quite distinct later on as shown in fig. 15 (Plate 28), for now the mesoderm comes to lie against the ectoderm and the endoderm very closely with a spacious coelom in between them. At the same time the mesoderm cells become spindle-shaped and lie stretched out between the two layers in the coelom. A further continuation of this process leads to the condition in fig. 16, where the entire mesoderm becomes mesenchymatous and invades the coelom, thus producing an irregular mass of cells lying between the ectoderm and endoderm. The primary coelom has ceased to exist.

On the ventral side the stomodæum appears as an invagination of the ectoderm, which comes to lie against the enteron and subsequently communicates with the latter. Thus the mouth opens into the stomodæum, which leads to the enteron. The latter opens to the exterior by the anus. The anus is not a new formation, as is the mouth, but is due to the direct transformation of the blastopore (fig. 17).

From now onwards there is agreement regarding the development of *Viviparus*. As Erlanger showed, from the mesenchymatous cells are produced two cavities—the right and the left pericardial cavities—which represent the “secondary coelom.” This view has been confirmed by later workers.

*Discussion.*

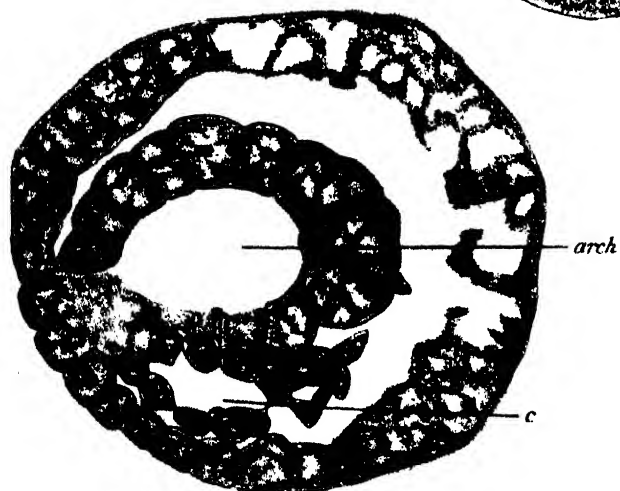
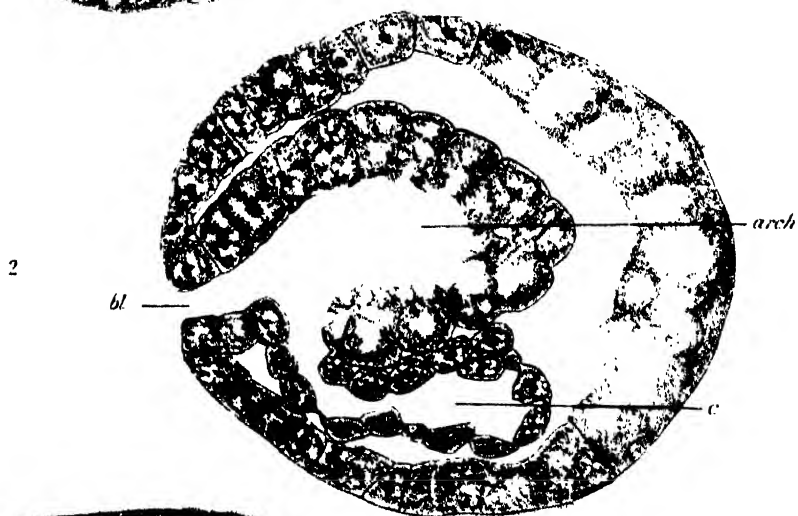
There are three methods of mesoderm formation in the Mollusca. In the enterocoelic method, which we have shown to exist in *Viviparus*, the mesoderm develops from a coelomic sac arising from the archenteron. The second is the teloblastic method where the mesoderm develops as a cell 4 *d* from the endoderm; this exists in the great majority of the Mollusca. According to the third method the mesoderm develops wholly from the ectoderm. This has been assumed for heavily-yolked eggs, but when we remember that Sarasin (1882) claimed it for *Bythinia*, and that Erlanger (1892) later showed that the method in *Bythinia* was teloblastic, we can definitely say that considerable doubt must be entertained as to the trustworthiness of all statements that go to prove the origin of mesoderm from the ectoderm.

The teloblastic and enterocoelic methods are related to one another. Cell 3 D in the former case corresponds to the archenteric wall of *Viviparus*, while cell 4 *d* in the former corresponds to the coelomic wall of the latter. Now in *Eupomatus*, the Annelid, we find an interesting connecting link. Here the gut is differentiated from cell 3 D; the larva swims for a day or two, after which period certain cells from the gut wall are budded out to give rise to mesoderm.

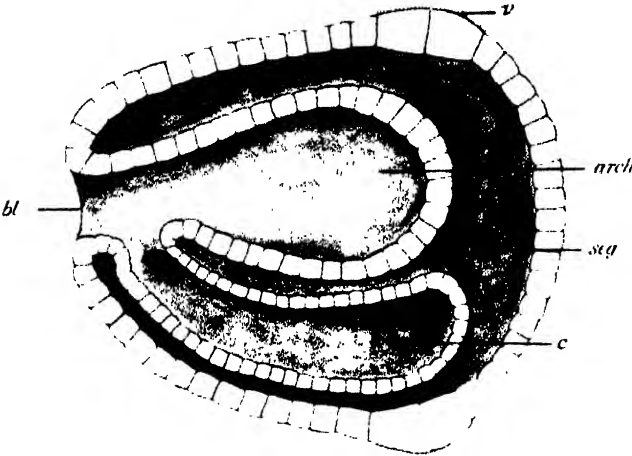
*Viviparus* shares with several other animals this method of development of mesoderm from a coelomic sac. It has been shown to exist in the Tardigrada, in the Echinodermata and in *Sagitta*. Caldwell showed that in the Actinotrocha the coelom arose from two pouches at the posterior end of the gut. We know that these types are widely separated from each other, but there is a singular set of features common to them all, namely, that the archenteron consists of a large number of small cells, the body cavity is moderately large and is not impeded by yolky cells surrounding it. If such a condition gives rise to a coelom, what is its significance? It has an ancestral significance, and with MacBride (1895) we have to agree that it shows that "the coelom appears to be, phylogenetically, simply a differentiated portion of the archenteron." No adults at the present day possess such a feature, but the larvæ of different phyla, when not impeded by yolk, recapitulate the stages from a Coelenterate ancestor to the present coelomate condition.

*Summary.*

1. In *Viviparus* segmentation is holoblastic and results in an invaginate gastrula.
2. At the posterior end of the archenteron a hollow sac grows into the blastocoel; this develops into the coelom.



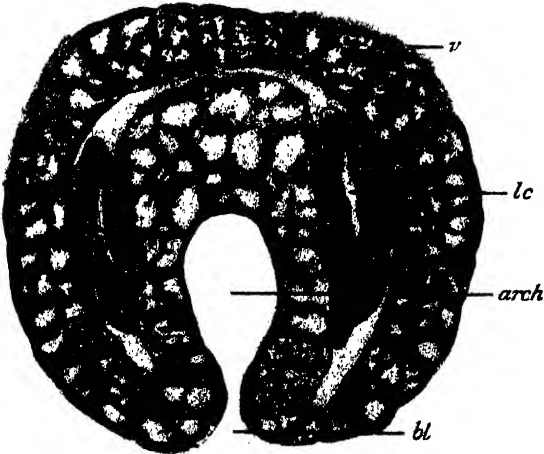


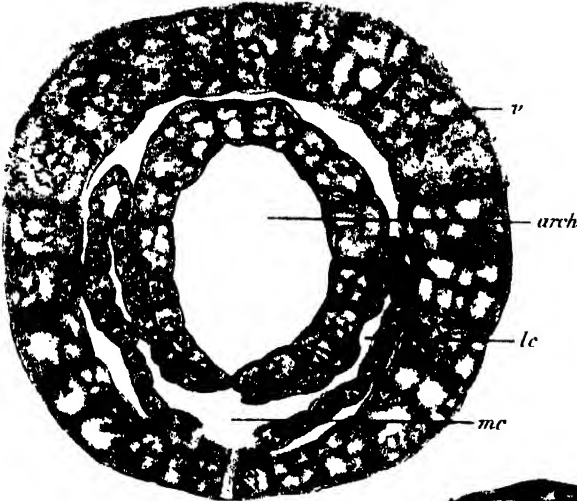


*lc*

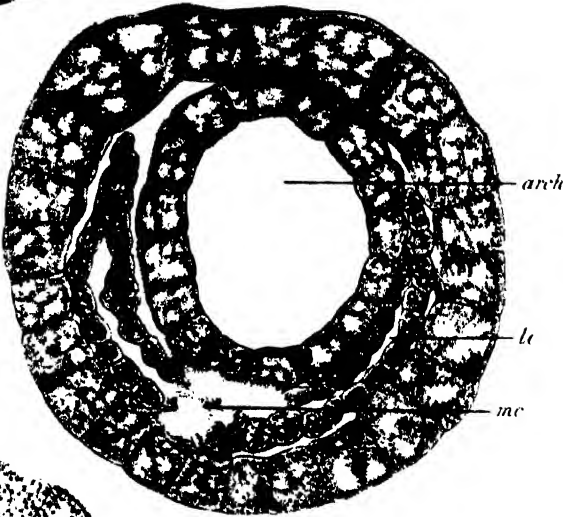
*-lc*

*-end*

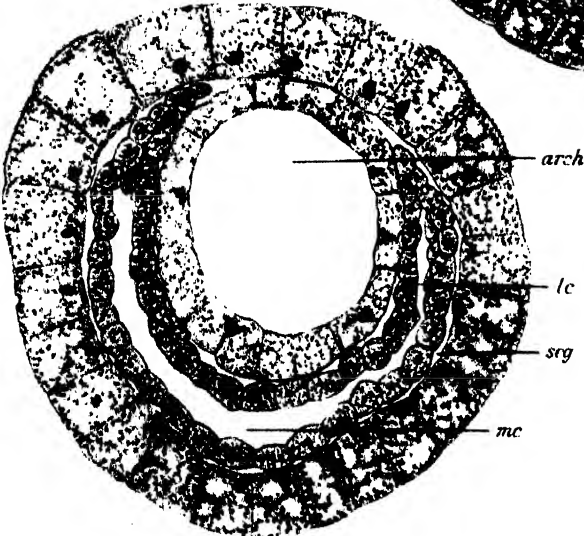




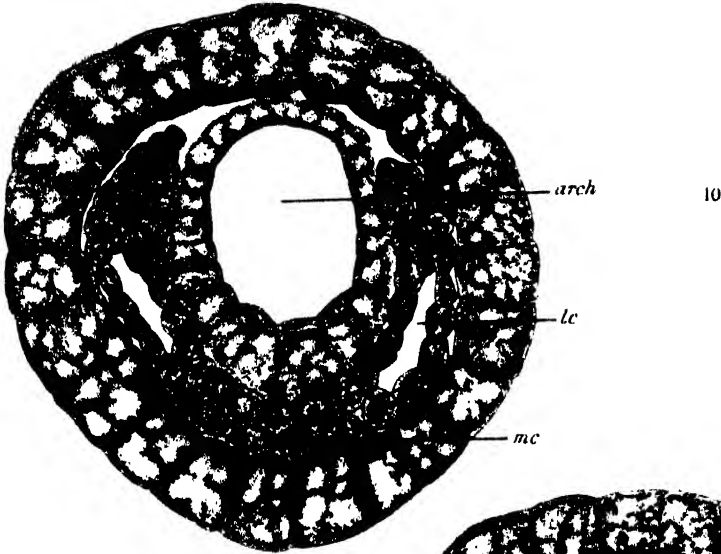
7



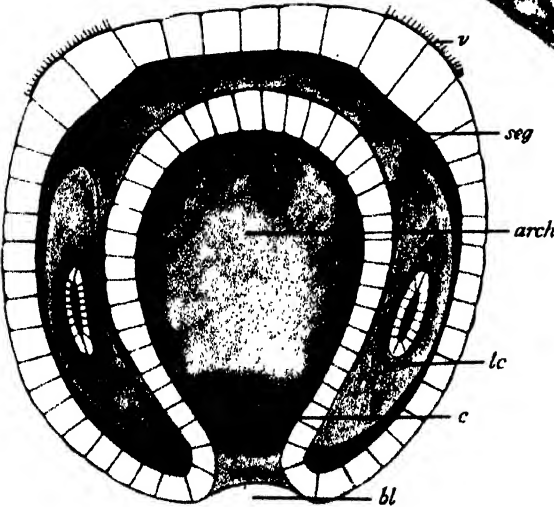
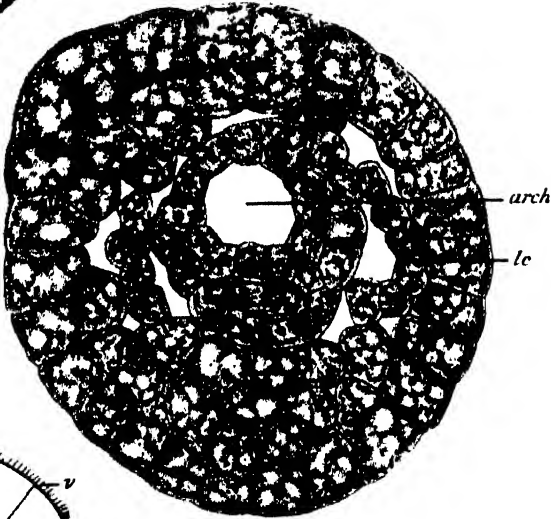
8



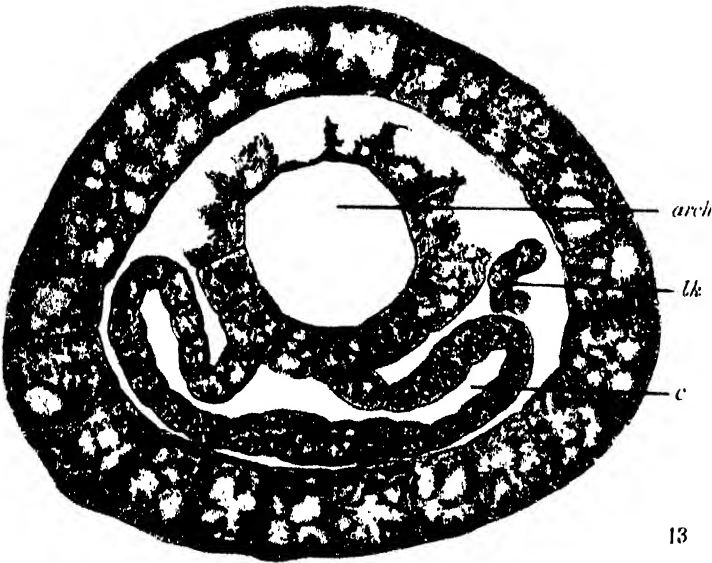
9



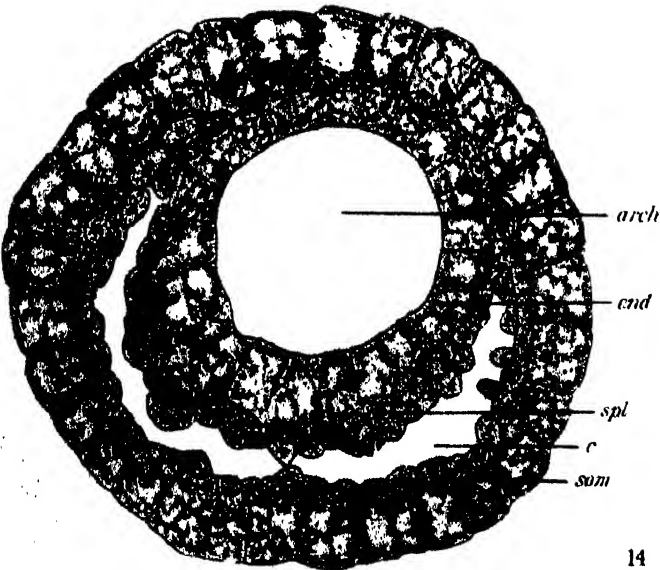
11



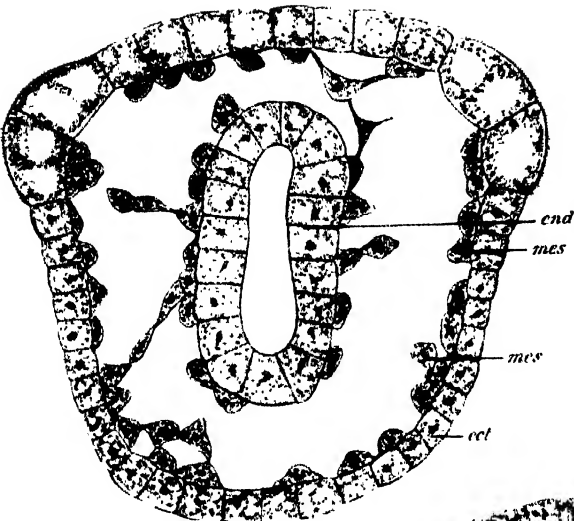
12



13

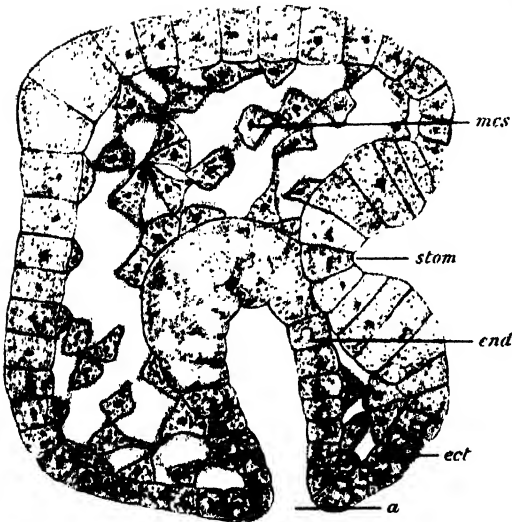
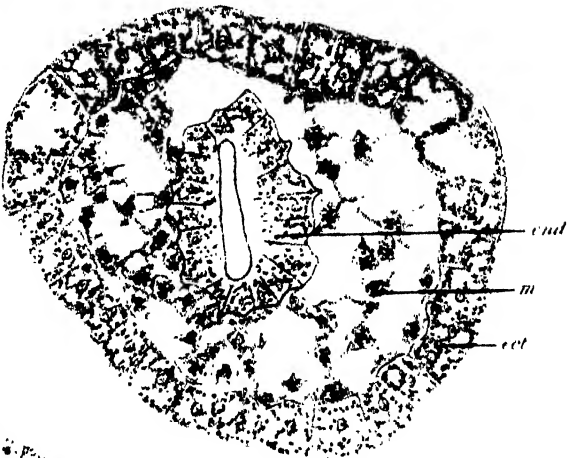


14



15

16



17

3. The coelom grows anteriorly and with the growth of the archenteron is pressed out laterally and dorsally.
4. The coelom becomes constricted off from the archenteron.
5. The walls break up and the cells are distributed irregularly in the blastocoel forming a mesenchyme from which later develop the typical "mesodermal organs."
6. The simple method of coelom formation is attributed to the lack of yolk, and has a phylogenetic significance.

#### LITERATURE.

- Bütschli, O. (1877). "Zur Entwicklungsgeschichte von *Paludina*," 'Z. Wiss. Zool.,' vol. 29.
- Dautert, E. (1929). "Die Bildung der Keimblätter bei *Paludina*," 'Zool. Jahrb. Anat.,' vol. 50.
- Erlanger, R. v. (1891). "Zur Entwicklung von *Paludina*," 'Morph. Jahrb.,' vol. 17.
- (1892). "Zur Entwicklung von *Bythinia*," 'Mitt. Zool. Stat. Neapel.,' vol. 10.
- (1894). "Mesoderm bei *Paludina*," 'Morph. Jahrb.,' vol. 22.
- Korschelt, E., and K. Heider (1893). 'Text-book of Embryology of Invertebrates,' vol. 4 (trans. by H. M. Bernard).
- MacBride, E. W. (1895). "Sedgwick's Theory of the Embryonic Phase of Ontogeny as an aid to Phylogenetic Theory," 'Quart. J. Micr. Sci.,' vol. 37.
- (1915). "Text-Book of Embryology," vol. 1.
- Otto, H., and C. Tönniges (1905). "Die Entwicklung von *Paludina*," 'Z. Wiss. Zool.,' vol. 80.
- Sarasin, P. (1882). "Entwicklungsgeschichte der *Bythinia*," 'Arbeit. Zool. Inst. Wurzburg.,' vol. 6.
- Tönniges, C. (1896). "Mesoderm bei *Paludina*," 'Z. Wiss. Zool.,' vol. 61.

#### EXPLANATION OF PLATES 23-28.

- FIG. 1.—Sagittal section of an early Trochophore passing lateral to the coelomic sac.
- FIG. 2.—Sagittal section of an early Trochophore passing through the blastopore, showing the communication between archenteron and coelom.
- FIG. 3.—Sagittal section of an early Trochophore showing coelom in its mid-ventral position.
- FIG. 4.—Section of a reconstructed early Trochophore to show the relation between the archenteron and the coelom.
- FIG. 5.—Dorsal region of a Trochophore in horizontal section.
- FIG. 6.—Horizontal section of a Trochophore passing through the blastopore and the archenteron.
- FIG. 7.—Horizontal section of a Trochophore showing the communication between archenteron and coelom. The coelom is represented by a median portion and two lateral portions.
- FIG. 8.—Horizontal section of a Trochophore showing the separation of coelom from the archenteron.

- FIG. 9.—Horizontal section of the ventral part of a Trochophore showing the cœlom lying in the segmentation cavity free from the archenteron.
- FIG. 10.—Horizontal section of a Trochophore passing through the wall of the median cœlomic sac. The archenteron has grown more posteriorly.
- FIG. 11.—Horizontal section of a Trochophore passing ventral to the cœlomic sacs, which are represented by a few cells.
- FIG. 12.—Reconstruction of a late Trochophore seen from dorsal surface. A portion of the dorsal half is removed.
- FIG. 13.—Transverse section of a Trochophore showing the cœlomic sac being detached from the archenteron.
- FIG. 14.—Transverse section of a Trochophore showing the cœlom entirely separated from archenteron. Cœlomic (mesoderm) cells are found on the dorsal surface also. The cœlomic wall is not of uniform thickness and cells begin to invade the cœlomic cavity.
- FIG. 15.—Horizontal section of a late Trochophore showing the mesoderm lining the ectoderm and endoderm and lying in the cœlom.
- FIG. 16.—Horizontal section of a late Trochophore showing the mesoderm producing mesenchymatous tissue.
- FIG. 17.—Sagittal section of a Trochophore showing the mesenchymatous tissue and transformation of the blastopore into the anus.

*Abbreviations.*—*a.*, anus ; *arch.*, archenteron ; *bl.*, blastopore ; *c.*, cœlom ; *ect.*, ectoderm ; *end.*, endoderm ; *l.c.*, lateral cœlomic sacs ; *l.k.*, larval kidney ; *mes.*, mesoderm ; *mesch.*, mesenchyme ; *m.c.*, median cœlomic sacs ; *seg.*, segmentation cavity ; *som.*, somatic mesoderm layer ; *spl.*, splanchnic mesoderm layer ; *stom.*, stomodæum ; *v.*, velum.

*The Origin and Development of the Pericardium and Kidneys  
in Ostrea.*

By WILFRED FERNANDO, B.Sc. (Lond.), Ceylon Government University  
Scholar, Marshall Scholar, Imperial College of Science and Technology.

(Communicated by E. W. MacBride, F.R.S.—Received October 8, 1930.)

[PLATES 29–32.]

*Introduction.*

The most complete account of the development of the pericardium, heart and kidneys in a Lamellibranch was given by Meisenheimer (1900) for *Dreissensia* [= *Dreissena*]. His conclusions were confirmed and extended to other Lamellibranchs by Harms (1909). Their work showed that the Lamellibranchs resemble other Mollusca as far as the later development of these organs are concerned, but the description of the early stages is very unsatisfactory; they assumed an ectodermal origin for the pericardium and kidneys, an idea which is inconsistent with what is known in other Mollusca.

It was suggested by Prof. E. W. MacBride, F.R.S., that the early development of the pericardium in *Ostrea edulis* be investigated with a view to determining the reasons which led Meisenheimer to uphold his old conclusions. My thanks are due to Prof. MacBride for his constant care and advice throughout the work; to Dr. R. W. Dodgson, Director of the Fisheries Experimental Station, Conway, for giving me all the facilities for obtaining the embryos and larvæ of *Ostrea* in his laboratory; to Mr. H. R. Hewer, and Mr. T. L. Green, for the help they have given me throughout the work.

*Technique.*

Unlike many other Mollusca, the eggs of *Ostrea edulis* cannot easily be artificially fertilised. But as this work did not necessitate a thorough working out of the cell-lineage, embryos obtained from the parent oyster proved quite satisfactory. A large number of oysters were opened and embryos of various stages of development were obtained from them. For the later development free-swimming veligers were taken.

The young embryos were fixed in Kleinenberg's picro-sulphuric acid, Mayer's



picro-nitric acid and Perenyi's chromo-nitric acid. By far the most satisfactory fixative was Perenyi's. For later stages Perenyi and Zenker were employed.

The embryos were embedded in cellodin and paraffin and stained in Delafield's hæmatoxylin.

#### *Description.*

Although there appears to be some confusion in his account of the early blastomeres, Horst's (1882) description of the early segmentation of *Ostrea edulis* agrees in principle with that given by Meisenheimer (1900) for *Dreissena*. As in the majority of other Mollusca the mesoderm originates from the macromere D. This gives rise to 2 D from which comes the future endoderm. The next division results in 3 D which buds out a small cell 4 d. This latter is the mesoderm mother cell, which divides into right and left halves (pole mesoderm cells). In the meantime the endoderm has been invaginated and forms a sac, the future enteron, and the pole mesoderm cells come to lie below the endoderm as represented in optical section in fig. 1 (Plate 29).

At the spot where the blastopore closes an ectodermal invagination, which comes into contact with the enteron, later forms the stomodæum. From the posterior end of the enteron is developed an outgrowth that tends to grow toward the lower pole of the embryo in the region of the mesoderm. This is the future intestine. The pole mesoderm cells have multiplied and on either side of the intestine produce a mesodermal strand. At the same time mesoderm cells are liberated individually from the bands and lie scattered about freely in the body cavity (fig. 2).

The cells in the mesodermal bands increase in number by repeated divisions, but the original pole mother cells remain larger than the rest and produce more mesoderm cells. The intestine, which at first was only a small outgrowth of the enteron, has now become elongated and is a distinctly tubular structure. During its subsequent growth it bends gradually in a line parallel with that of the stomodæum and approaches the ectodermal wall (fig. 3).

As it elongates thus it comes to lie between the two mesodermal bands and pushes to either side the original pole mesoderm cells (fig. 4, Plate 30).

The shell gland begins to develop at this stage and form the bivalve shell (*sh.*) which appears on both sides in a frontal section. More mesoderm cells are liberated into the body cavity, but in the mesodermal bands (*mes.*) the pole mesoderm cells (*pm.*) still remain larger than the rest.

By repeated divisions the pole mesoderm cells gradually lose their distinctness and the mesoderm becomes a compact mass on each side of the intestine. The mesoderm cells are spherical and at this stage are quite different

from either ectoderm or endoderm cells. More cells are given off into the body cavity and these become elongated; they constitute the future mesenchyme cells (*mesch.*, fig. 5).

Following this stage an invagination occurs in the ectoderm at the posterior pole of the embryo. This invagination is the proctodæum, which soon comes into communication with the intestine, and the whole alimentary canal is differentiated. Below the anus is developed a tuft of cilia, the telotroch.

So far the mesodermal bands appear to be masses of undifferentiated cells; they lie on both sides of the intestine in the region of the "rectum." A reconstruction of a larva at this stage is shown in fig. 6. The mesodermal bands are small in size by comparison with earlier stages, since most of the cells are liberated as spindle-shaped mesenchyme cells which are characteristic of the embryos. The mesodermal band itself is now differentiated into two regions—a lower portion which represents the main mesodermal band and an upper portion which appears to become constricted off from the lower part. This upper mass of mesodermal cells lies at the side of the rectum.

Fig. 7 (Plate 31) represents a frontal section of the trochophore. It shows a well-developed prototroch. The œsophagus (*oes.*) lies in the dorsal portion of the body cavity; below it is a stained mass in which no distinct nuclear structure is apparent. In the ventral region is the rectum (*rect.*) on each side of which is the mesoderm. A part of the mesoderm (*mes.*) lies in the body-cavity, while a smaller portion (*per.*) lies next to the rectum. This latter portion corresponds to the upper portion of the mesoderm mass in fig. 6. This mass of mesoderm on either side of the rectum is the rudiment of the pericardium and the kidneys. Even at this early stage these cells are characterised by a deeper staining capacity. While it forms a part of the mesodermal band, it, at the same time, comes to lie on the outer ectoderm, thus tending to give the impression that it is part of the ectoderm. But there is no difficulty at all, in this stage, in seeing its mesodermal origin, for the shape, size and appearance of the cells is distinctive. Further, in *Ostrea*, the cells are large and the cell boundaries distinct which renders easy the identification of the various cells. The true relationships of the various cell masses become quite apparent in a reconstruction.

The pericardial rudiment (*per.*) is soon cut off from the rest of the mesoderm (*mes.*). It then looks as if it was a product of the ectoderm, while the remaining mesoderm cells become elongated and are scattered about as mesenchyme. These cells come to lie in the body cavity between the enteron and the ectoderm. They are later transformed into muscle and the connective tissue of the adult.

The beginning of all these processes is seen in the older trochophores (fig. 8).

The embryo is still in the trochophore stage and its shell gland is well developed. The isolation of the pericardial rudiment is now complete (fig. 9) and it is so closely applied to the ectoderm that it appears to be ectodermal in origin. In fact if one begins with a stage like this the ectodermal nature of the pericardium appears to be obvious.

A velum is developed and the trochophore now becomes a veliger. The bivalve shell has grown considerably and covers all the internal body structures. Fig. 10 (Plate 32) is a part of the frontal section of a veliger. On the outside is the bivalve shell, lining the mantle fold. The rectum (*rect.*) is in the lowest region. On each side of it is the mass of cells which gives rise to the pericardium and kidneys. This mass now appears to be continuous with the mantle lining (*m.*). More dorsally are seen the lobes of the liver (*l.*) and a free mesenchyme cell (*msch.*) also appears in the body-cavity. From the pericardial mass cells are given out in two directions. Some go towards the region of the liver (*k.*). These produce the kidneys, the one on the left side has already begun to form a cavity. Other cells (*per.*) travel towards the rectum. These at first form solid masses, the rudiments of the pericardium.

During further growth (fig. 11) in each of the pericardial rudiments a small cavity is distinguishable. These two cavities (*per.*) lie above (morphologically below) the rectum and seem almost to surround it. The kidneys have in the meantime developed, but still no connection appears between them and the pericardial cavities. So that it is clear that primarily no connection exists between the kidneys and the pericardium, communication being effected at a later stage.

The pericardial cavities increase in size and soon meet each other in the middle line below the rectum. With the disappearance of the partition wall a single pericardium appears lying immediately below the rectum as in the adult (fig. 12).

#### Discussion.

There are two views regarding the origin of the pericardium, kidneys and gonads in Mollusca with special reference to the Lamellibranchia. One view is that the above organs are products of the mesoderm; the other, which is directly opposed to the former, is that it is purely ectodermal in origin.

Ziegler (1885) showed that in *Cyclas* [= *Sphaerium*] the pericardium arises from the anterior part of the mesodermal bands. In this mass a cavity appears and by later fusion of these cavities a pericardium results. Ziegler's account is

in perfect accordance with what was already known in other forms, namely, that the blood vascular system, kidneys and gonads were mesodermal products.

Meisenheimer (1900), in his description of the development of *Dreissena*, gives an entirely different origin for the coelomic organs. According to him there are pole mesoderm cells in *Dreissena* just as in other Mollusca, but these do not give rise to mesoderm, and the pericardial rudiments arise distinctly from ectoderm. Therefore the Lamellibranch pericardium is entirely different from the mesodermal pericardium of other Mollusca. Meisenheimer (1898) upheld a similar idea for the Pulmonate *Limax*. To prove this conclusion Meisenheimer (1901) next worked on *Sphærium* and contended that Ziegler's account was erroneous and that the pericardium arose from a part of the ectoderm near the intestine where the anus later develops.

About the same time Ahting (1901) described the development of the heart and kidneys in *Mytilus*. He confined his attention to the later development, but he stated that he was able to find that the origin of the above organs could be traced to the mesoderm, for his early stages corresponded to Ziegler's diagrams which showed the mesodermal bands. Since Ahting did not trace back the origin to the primary mesoderm cells his views were naturally questioned.

Harms (1909) then worked on the Unionidæ and found that he was in entire agreement with Meisenheimer with regard to the ectodermal nature of the pericardium. He says "Sie stellt eine paarige ectodermale Wucherung dar, die in hinteren Theile des Embryos bzw. der Larva jederseits von Enddarme gelegen ist."

Finally Herbers (1913) repeated the work on *Anodonta* and his conclusion is: "Im Verlauf der Anodonta-Entwicklung gehen Nieren, Herz, Pericard und Geschlechtsorgane aus einer paarigen, gemeinsamen Anlage hervor, die beiderseits in Form von Streifen in hintersten Abschnitt der junger Larven ruhen. Diese Streifen leiten sich nicht vom Ectoderm ab, sondern sind direkt auf die aus dem primären Mesoblasten entstandenen Mesodermstreifen zuruckzufuhren."

The present work is a confirmation of Ziegler's statement for *Sphærium* and Herber's for *Anodonta*. Meisenheimer and Harms had mistaken for true ectoderm the mesodermal mass which lined the ectoderm. But we must examine carefully the evidence adduced by Meisenheimer from his sections. All the figures in this paper were of frontal sections and they show that the mesodermal band lie on each side of the intestine and that the pericardial

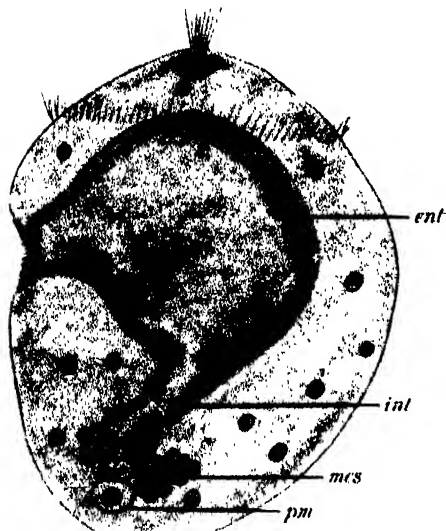
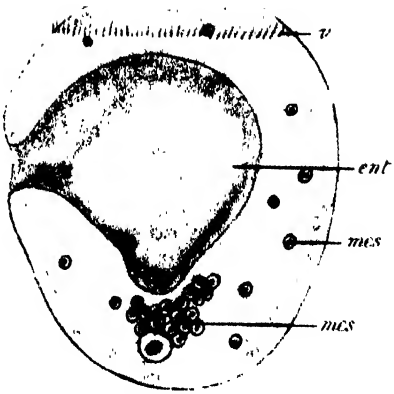
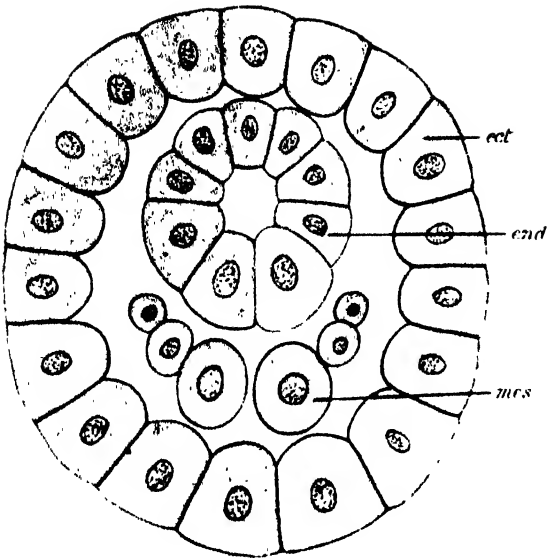
rudiment likewise is situated on both sides of the rectum. Harms also admits that the rudiment of the pericardium lies in this position. This being the case, it will be impossible to obtain in a sagittal section both the pericardial rudiment and the rectum. But Meisenheimer (1900) figures sagittal sections through young larvæ where he is able to show the pericardial rudiment and the rectum (fig 70, p. 130). If Meisenheimer's interpretation of this rudiment were correct we should expect it to lie behind the intestine, in which case a frontal section of the larva will not reveal both the rudiment and the rectum. However, Meisenheimer's sections of later stages, which correspond to our frontal sections of late stages, are cut frontally. We infer therefore that Meisenheimer's interpretation of his supposedly ectodermal pericardial rudiment is not correct.

On examination of Meisenheimer's description of the development of the pericardium in *Sphaerium* (fig. 1) and that of Harms (fig. 6A, p. 7) it appears probable that both of them started with a stage corresponding to that shown in the present paper as fig. 9. We have shown that in this stage the ectodermal nature of the pericardial rudiments is a superficial appearance, because it is only in these later stages that the mesoderm comes to lie against the ectodermal wall; in earlier stages the cell boundaries are distinct, in later stages they are obscure causing difficulties in interpretation. A similar error has been committed by Tönniges in *Viviparus*.

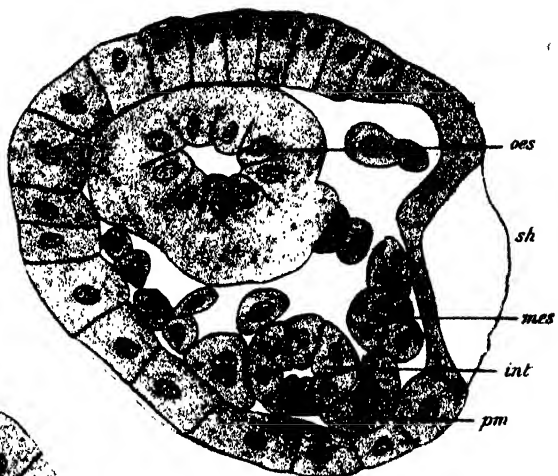
If the pericardium in Lamellibranchs is ectodermal in origin it is an entirely different organ, not homologous with that of other allied groups, a view which is hard to believe in face of the evidence of comparative adult morphology alone.

#### *Summary.*

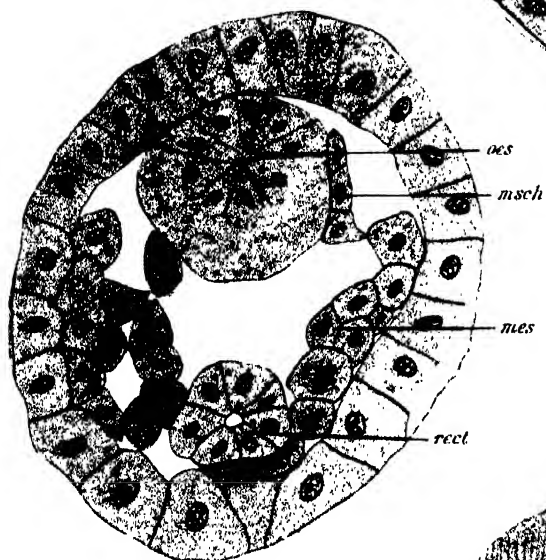
1. The pole mesoderm cells divide to produce two mesodermal bands.
2. A part of each band becomes differentiated and lies at the side of the rectum. The rest produce connective tissue and muscle.
3. The portions which lie next to the rectum are detached from the rest of the mesoderm and come in contact with the ectoderm.
4. From the masses on the sides of the rectum are differentiated the kidneys and the pericardium.
5. A cavity appears in each pericardial mass.
6. The two cavities meet in the middle line below the rectum and produce the ventral pericardium of the adult.
7. The probable reason for Meisenheimer's and Harms' misinterpretation



4

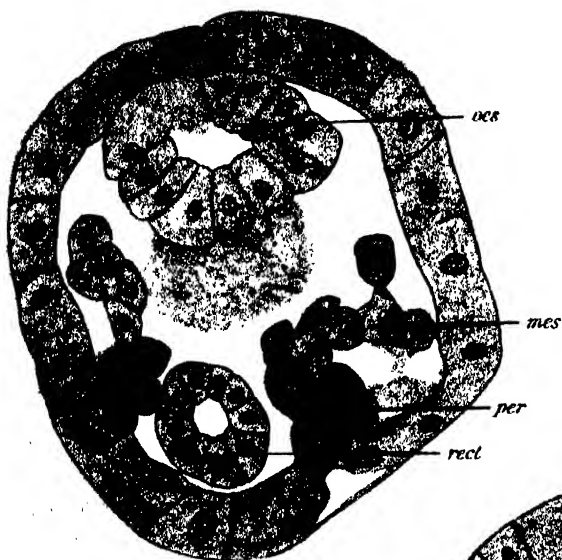


5



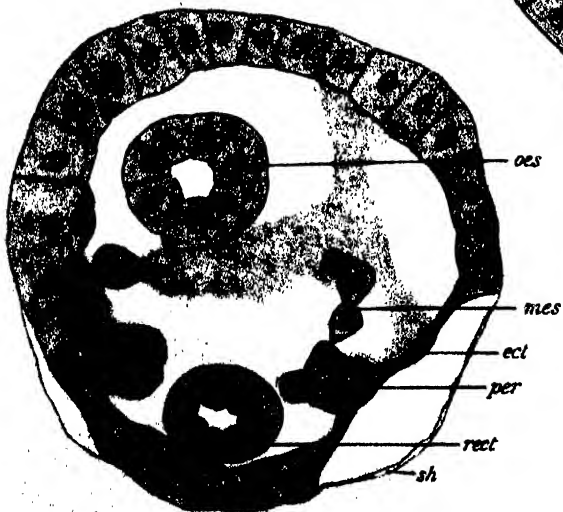
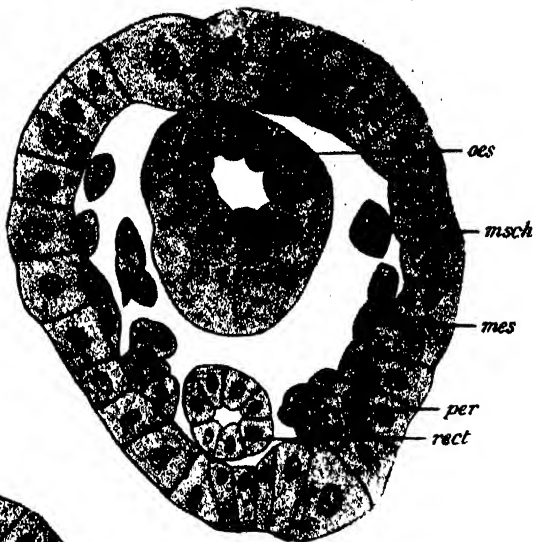
6





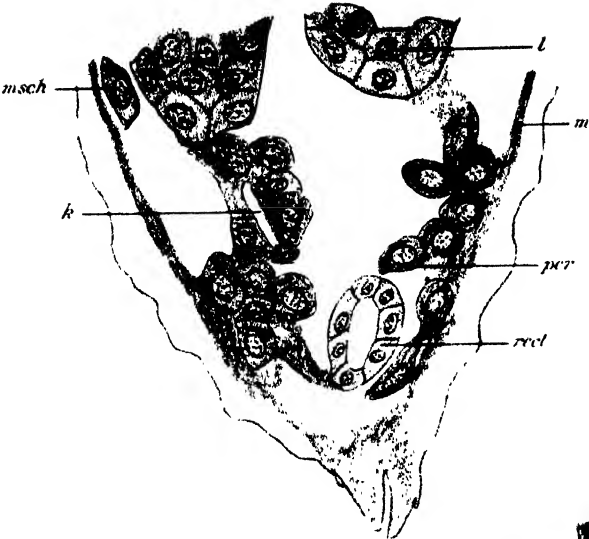
7

8

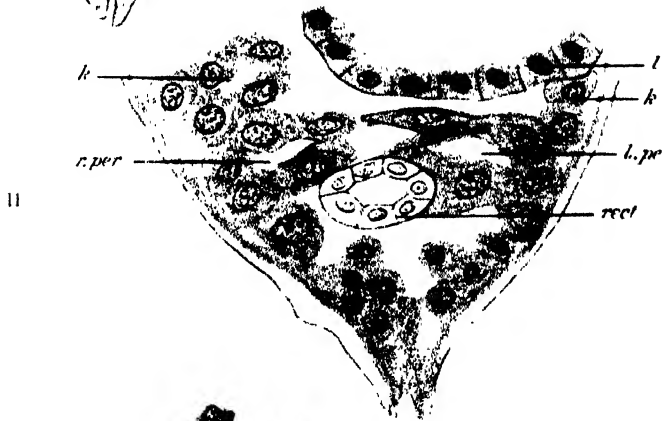


9

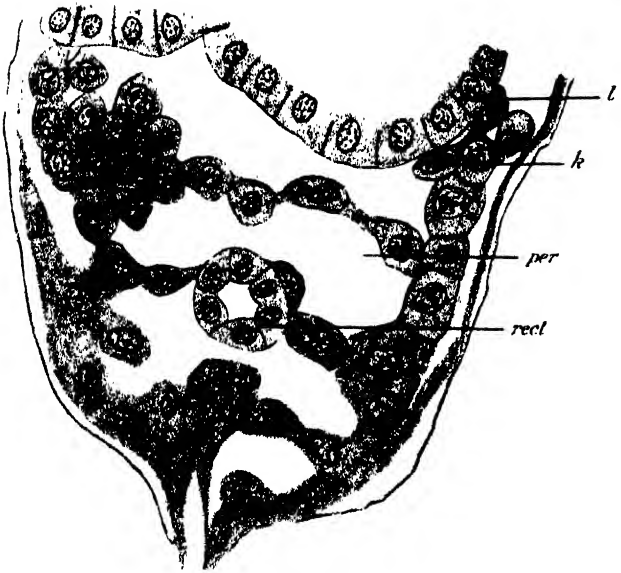




10



11



12

is that they started with the later stages when the mesodermal mass lies on the ectoderm.

8. The mesodermal nature of the pericardium in Lamellibranchia brings this group into line with other Mollusca.

#### LITERATURE.

- Ahting, K. (1901). "Die Entwicklung des Bojanus'schen Organs und des Herzens der Lamellibranchier," 'Jena Z. Naturw.,' vol. 36.
- Harms, W. (1909). "Postembryonale Entwicklungsgeschichte der Unioniden," 'Zool. Jahrb.' (Anat.), vol. 28.
- Herbers, K. (1913). "Entwicklungsgeschichte von Anodonta," 'Z. Wiss. Zool.,' vol. 108.
- Horst, R. (1882). "On the development of the European Oyster," 'Quart. J. Micr. Soc.,' vol. 22.
- Meisenheimer, J. (1898). "Entwicklungsgeschichte von *Limax maximus*. II. Die Larvenperiode," 'Z. Wiss. Zool.,' vol. 63.
- (1900). "Entwicklungsgeschichte von *Dreissensia*," 'Z. Wiss. Zool.,' vol. 69.
- (1901). "Die Entwicklung von Herz, Pericard und Genitalzellen bei *Cyclas* im Verhältnis zu den übrigen Mollusken," 'Z. Wiss. Zool.,' vol. 69.
- Ziegler, H. E. (1885). "Entwicklung von *Cyclas cornea*," 'Z. Wiss. Zool.,' vol. 61.

#### EXPLANATION OF PLATES 29-32.

- FIG. 1.—Frontal section of an Embryo.
- FIG. 2.—Side view of an Embryo showing the mesodermal band and the outgrowth from the enteron.
- FIG. 3.—Side view of an Embryo showing growth of the intestine posteriorly.
- FIG. 4.—Frontal section of an Embryo.
- FIG. 5.—Frontal section of an Embryo showing development of mesenchyme from the mesodermal band.
- FIG. 6.—Side view of an Embryo showing the differentiation of the mesoderm.
- FIG. 7.—Frontal section of an Embryo showing the relation of the pericardial rudiment to the ectoderm and the general mesoderm.
- FIG. 8.—Frontal section of an Embryo at a stage later than in fig. 7.
- FIG. 9.—Frontal section of an Embryo showing the isolation of the pericardial rudiment from the remaining mesoderm, and its attachment to the ectodermal layer.
- FIG. 10.—Part of frontal section of a free-swimming Veliger showing the differentiation of the kidneys and pericardium from the original mesodermal mass.
- FIG. 11.—Part of frontal section of a Veliger showing the developing pericardial cavities.
- FIG. 12.—Part of frontal section of a Veliger near metamorphosis showing the fusion of the right and left pericardial cavities to produce the ventral pericardium.

*Abbreviations.*—*a.*, anus; *ect.*, ectoderm; *end.*, endoderm; *ent.*, enteron; *int.*, intestine; *k.*, kidney; *l.*, liver; *l. per.*, left pericardium; *m.*, mantle wall; *mes.*, mesoderm; *mesch.*, mesenchyme; *oes.*, oesophagus; *per.*, pericardial rudiment; *p.m.*, pole mesoderm cell; *r.*, right pericardium; *rect.*, rectum; *sh.*, shell; *tel.*, telotroch; *v.*, velum.

CROONIAN LECTURE.—*The Theories of the Bacteriophage.*

By JULES BORDET, For. Mem. R.S.

(Lecture delivered December 11, 1930—MS. received November 6, 1930.)

Allow me, in the first place, to tell you what pleasure I experienced when the Royal Society, now many years ago, elected me to its Foreign Membership, and how deeply I feel to-day the honour of having been chosen to give to the Society the Croonian Lecture for this year.

The phenomenon of the destruction of microbes known under the name of "bacteriophagy," or transmissible autolysis, quickly aroused the liveliest interest, firstly, because its importance was foreseen, and secondly, and perhaps to a greater degree, by reason of its character, so bizarre and so unexpected. Is it not, indeed, surprising to find that a bacterial culture, grown on the surface of an agar medium, becomes sprinkled with holes, in the neighbourhood of which the culture is deeply corroded? Is it not, further, remarkable to find that a broth, to which has been added the active principle responsible for this effect, is thereby rendered unsuitable as a medium for culture, or that, under the action of the same principle, a turbid microbial suspension, obtained by emulsifying the culture from a solid medium, becomes clear? Is it not even more remarkable that the active principle regenerates itself in producing its effect, and can accordingly be reproduced indefinitely?

The fundamental observations are very striking. It is easy to obtain the active principle; one often finds it in water or in soil, and very frequently in faecal materials. If these latter are diluted with water or bouillon, and passed through a filtering candle, they often prove to be so powerfully lytic, especially for the bacillus of dysentery, that it suffices to add a trace of the filtered liquid to a suspension of this bacillus, in order to produce quickly an obvious lysis. Having obtained this effect, d'Hérelle showed that this suspension, in which this lytic effect had appeared, furnished, on filtration, a liquid of which, again, a trace would suffice to produce lysis in a new suspension of the dysentery bacillus, and so on, in indefinite sequence. One of your countrymen, a Fellow of this Society, Twort, had previously recorded an observation which did not attract much notice at the time, but which, as I thought necessary to mention, presents a fundamental analogy to the experiment of d'Hérelle. Vaccine lymph very frequently contains a principle of this kind acting on the staphylococcus. If one spreads such a lymph on agar,

colonies develop, among which there are some which soon manifest a very intense lysis. The colony fades and the microbes in it break down into invisible débris. Then, if one emulsifies such a lysed colony in a little bouillon and filters the mixture, the liquid obtained, when brought into contact with normal staphylococci, produces lysis of these. By a whole series of experiments Gratia proved the perfect identity, in every respect, between the phenomenon of Twort and the phenomenon of d'Hérelle.

It was soon recognised that there exists a number of these principles, distinguished from one another by the species of microbes which each has the power of attacking. For example, a principle acting on the staphylococcus does not produce any effect on the *Bacillus coli*, or on the dysentery bacillus. One often finds, however, that the same active principle can attack several microbial species, if these are sufficiently related. In particular, there exist principles acting on *Bacillus coli*, which are also capable of affecting the dysentery bacillus. In such a case, namely, when a principle specific for a certain species is brought into contact with another species of microbe, one usually finds that its virulence for the latter becomes enhanced with successive transmissions. Suppose, for example, that we introduce into a suspension of dysentery bacilli a principle acting on *Bacillus coli*. A lysis, more or less intense, will be produced. After two or three days in the incubator, we filter this suspension and add a little of the filtrate to a new suspension of the same dysentery bacillus. We shall observe a more pronounced lysis than at the first application. One may accordingly conclude that the principles are susceptible of adaptation. In this respect, however, each of the different principles has its own characteristic behaviour. The specificity of the principles and their power of adaptation are very variable. The principles are destroyed by heating to a temperature below 100°, frequently in the neighbourhood of 70°. These preliminary data being established, let us recall to memory a few very simple experiments. Having obtained a principle acting on the colon bacillus, we inoculate a tube of bouillon with some drops of a culture of this microbe, and add a very small dose of the lytic principle. Immediately afterwards we spread a drop of the mixture on the surface of agar in a tube. We repeat the same operation at certain intervals; for example, at the end of 1 hour, 2 hours, 4 hours and 6 hours, the different agar tubes thus inoculated being, of course, put into the incubator. If the dose of the principle was sufficiently weak, one finds that the first agar tube, inoculated with a drop taken immediately after the principle was added, becomes covered with a continuous and normal layer of bacterial growth, and does not become sprinkled

with the clear spots which are characteristic of the lysis. The agar tube, however, which was inoculated an hour after the principle had been added, shows a number of such spots, and those inoculated after 2, 4 and 6 hours show them in still greater numbers. This result proves that the lytic principle multiplies itself in the culture of *Bacillus coli*.

On the other hand, let us emulsify in broth a culture of *Bacillus coli* obtained on agar, and let us distribute the suspension so obtained in equal quantities in a number of sterile tubes, and let us introduce into these, different quantities of the lytic principle. Immediately after this addition, let us take from each tube one drop, which we spread on agar, and place the agar tubes in the incubator. The *Bacillus coli* grows, but the surfaces of the different cultures soon show a sprinkling of lytic spots, which are more numerous in proportion to the increasing amounts of the lytic principle, added to the suspensions from which the culture-tubes were inoculated.

It was on these experiments, essentially, that d'Hérelle based his virus-theory of the bacteriophage, according to which the lysis is attributed to a parasite, so small that it can pass through bacteria-proof filters. This virus penetrates into the bacterium and soon causes its destruction. The spots of lysis, with which the surface-cultures on agar become sprinkled, thus represent actual colonies of virus, or, more precisely, colonies of infected bacteria, which the virus soon destroys. Just as by spreading a dilute suspension of bacteria on agar, one obtains a number of colonies which is greater in proportion to the greater richness of the suspension in organisms, in the same way a bacterial suspension, to which the lytic principle has been added, gives an increased number of spots of lysis in proportion to an increase in the quantity of the principle added, that is to say, in proportion to the increase in the number of virulent units. At every point where a virulent unit has been deposited, it multiplies itself while attacking the neighbouring bacteria. The fact that, when the dilution of the principle is adequate, the spots of lysis are well defined and isolated, is used by d'Hérelle as evidence in favour of the virus-theory, since it proves that the virus, when spread on agar, is deposited on certain points of the nutrient surface and not on others—that is to say, that it is present in a corpuscular form, not in a state of solution. In reality, the conception of the principle being a very small corpuscle does not strictly involve its being a virus. The principle loses its activity when heated to about 70°. It is very probably a colloid, and colloids are composed of particles. It is also possible that it may be adsorbed by the bacterial débris resulting from lysis, and that it is thus deposited with these débris at certain points on the agar

surface. Moreover, even if the principle, instead of being in a corpuscular form, were in a state of solution, the phenomenon of the isolated spots of lysis would nevertheless be observed. Whether the principle is a virus or not, it is certain that contact with the bacteria sensitive to its action is necessary for its reproduction. Let us suppose that one introduces gradually diminishing volumes of the principle into a series of microbial suspensions. When the dose is thus reduced, one arrives at an extremely small quantity of the principle, only just above that which would produce no effect at all. Thus, when the principle acts in such an extremely weak dose, whether it is a virus or an active substance in solution, it is legitimate to suppose that it will not be able to affect more than a small number of the bacteria present. It is well known, indeed, that all the individual microbes in a culture are not absolutely identical, and it is highly probable that they do not all exhibit an absolutely equal sensitiveness to the action of the lytic principle. As a matter of fact, it can be demonstrated, in comparing the effects of equal concentrations of the same principle on two bacterial species which are unequally sensitive to it, that the culture obtained, by spreading on agar, from the more sensitive microbe will present the greater proportion of spots of lysis. But when one bacterium is attacked by the lytic principle, it reproduces it, it becomes a centre for the regeneration of the agent. The latter, as a consequence, becomes much more abundant in the immediate neighbourhood and can thence attack more resistant individuals. As a consequence, on each point of the agar medium on which a bacterium has been deposited, which is a little more sensitive to the principle than the others, a spot of lysis will appear. If the suspension spread upon the agar only contains a very small quantity of the principle, the phenomenon of the isolated spots must inevitably be produced, no matter what may be the state of dispersion in which the principle exists. (The filtration experiments carried out by different authors appear to show that the molecular or micellar dimensions of the principle are analogous to, if not smaller than, those of the colloidal particles of different albuminoids.)

The virus-theory of d'Hérelle appears truly seductive, by reason of its great simplicity. It is well known that various objections were soon raised to it, notably in relation to the extraordinary resistance of the principle to storage and to the action of certain antiseptics, such as chloroform. It preserves its activity when kept in a sealed tube in presence of an equal volume of chloroform. After being kept for a year or more, the principle develops its effects as quickly as one freshly prepared. If it were a virus, one would expect the principle to require an appreciable time for the recovery of its vital activity,

after such a long period of inaction. When one studies the mode of action of these principles, one meets with various facts which are difficult to reconcile with the hypothesis of an intrabacterial virus. I have pointed out, in particular, that, if one introduces a very weak dose of the principle into a tube of bouillon, inoculated immediately before with the sensitive organism, the latter multiplies during the first few hours as actively as if the medium contained none of the principle. But afterwards, at a given moment, after 4 or 5 hours, for example, the liquid which had become turbid clarifies itself with an extraordinary suddenness, as if all the microbes underwent lysis almost at the same time. It would be difficult to suppose that an intrabacterial virus, originally present in small amount and at first allowing the microbes to develop, attacks them all, at a given moment, almost simultaneously. It would, in fact, be easier to reconcile the phenomenon with the hypothesis of an extrabacterial virus, which should secrete a diffusible ferment digesting the bacteria, the latter not undergoing lysis until the concentration of this ferment in the liquid became sufficiently high. But d'Hérelle has shown that the principle cannot be cultivated at all in the uninoculated bouillon, and, further, that it does not develop in bouillon to which killed organisms have been added. The microbes must be living. Actually, the later researches of Doerr and Gruninger have proved that, under the conditions above indicated, namely, when one inoculates with the sensitive organism a bouillon containing only a trace of the principle, the quantity of the latter increases during the first few hours, while the bacteria are multiplying. The lysis does not become effective until the content of active principle has been considerably raised; it is then accomplished, as has just been indicated, with a remarkable promptitude. This last observation seems clearly to indicate that the regeneration of the principle is correlated, not strictly speaking, with the intensity of the lysis, but with the bacterial multiplication which precedes the lysis; and one cannot help thinking that this fact is favourable to the theory which I have proposed 10 years ago with Ciuca, and according to which it is the bacterium itself which secretes the lytic principle—that is to say, that bacteriophagy is, in reality, an autolysis. Moreover, the idea that the bacterium plays a very active part in the production of the lytic principle arises also from a fact which I established with Jaumain. This fact is that the regeneration of the principle requires not only that the sensitive bacteria shall be living, as d'Hérelle had already indicated, but also that they shall be nourished, that is to say, that they shall be able to multiply. If one emulsifies the bacteria, not in bouillon, but in physiological saline, which has no nutritive properties, and if one introduces the principle into this sus-

pension, one observes neither lysis of the organisms nor regeneration of the principle. But to cause these phenomena to appear, it is sufficient to add a little peptone or meat extract. If one introduces a little of the principle into a suspension in bouillon which contains too great a quantity of the bacteria, this very strongly turbid liquid remains opaque. But, in order to obtain quickly an almost complete clarification, it is sufficient to add a supplementary volume of bouillon.

In 1921 I observed, with Ciuca, that if one injects a lysed bacterial suspension into a rabbit, the animal soon gives an antilytic serum, that is to say, a serum which, in a sufficient dose, completely neutralises the lytic activity of the principle and finally suppresses its power of regenerating itself. We have observed, moreover, that the serum retains its activity when it is heated to 60°, a temperature which spares such antibodies as the antitoxins, but abolishes or greatly reduces the bactericidal properties of sera. If we were dealing with a virus, it seems that it ought not to be destroyed by such a heated serum, while exhibiting such a good resistance to long storage and even to chloroform.

Stassano and de Beaufort had found that the addition of sodium citrate hinders the lytic effect of a principle. A little later I demonstrated that, at least for certain principles, the elimination of calcium, produced by adding about one part of neutral sodium oxalate per thousand to the bacterial suspension, not only suppresses the lysis in bouillon, but also hinders the regeneration of the principle, although the sensitive bacteria are tolerant of the calcium privation, develop perfectly, and should still be able to serve for the nourishment of the virus. There are, indeed, other principles, of which the lytic action and the power of self-reproduction are not thus adversely affected by oxalate, but we found certain principles which behave, if we may so express it, in an intermediary fashion, in the sense that, in the presence of oxalate, they only produce a very slow and incomplete lysis, and only slowly reproduce themselves. It is therefore permissible to suppose that all the principles require some calcium for their action and reproduction, but that they do not all require the same dose of it, some needing quite a strong concentration of calcium, while others are content with such traces as oxalate does not succeed in precipitating completely.

The impression resulting from these considerations is not at all favourable to the hypothesis of an intrabacterial virus. However, if one does not shrink from the supposition that a living organism tolerates contact with such agents as chloroform, and if one can further conceive that a parasite requires that the bacteria, of which it makes its prey, be not only living, but further have at



their disposal the nutritive materials necessary for their reproduction, the facts reviewed above do not irrevocably condemn the idea of a virus, destroying bacteria by means of an enzyme which often requires the presence of calcium salts for its action. However that may be, I have proposed with Ciuca, in 1920, a theory which is frankly opposed to that of d'Hérelle, and which may be formulated as follows :—

1. The invisible virus of d'Hérelle does not exist. It is the bacteria themselves, subjected to the lysis, which reproduce the lytic principle. Bacteriophagy is accordingly a case of autolysis.
2. The intense lytic action, to which the name bacteriophagy is given, represents the pathological exaggeration of a function belonging to the physiology of the bacteria. In other words, bacteriophagy is the result of a disturbance which has occurred in the accomplishment of a normal process.
3. This normal function, which acts in some way as the source from which the phenomenon of bacteriophagy originates, may have some relation to the phenomena of mutation. It is established that individual microbes, belonging to one and the same species, may exhibit appreciable differences among themselves. Within the borders of a single species distinct types may become constituted, and an equilibrium may be established between them, the maintenance of which could not easily be explained, unless corrective influences intervened, in order somehow to discipline the evolution of the species, by repressing the tendency of certain strains to obtain an excessive preponderance, and thus permitting the appearance or the survival of other varieties. It is in this regulating mechanism, which assures to the species its normal destinies and evokes the idea of an antagonism between the different types, that one should probably seek for the factors originally determining the appearance of these phenomena of rapid bacterial destruction, which the study of bacteriophagy has revealed. To summarise, the co-existence of different types in a single species justifies us in believing that mutual reactions between them can occur, and one may imagine that, as the result of certain circumstances, these reactions become considerably exaggerated, and thenceforward represent a cause of destruction for the microbes, that is to say, they acquire a frankly pathological character.

One perceives at once the difficulties which must be surmounted before such a conception, which at first appears purely hypothetical, can take rank with positive ideas, solidly based on facts. In the first place, an essential character of the bacteriophage is to be transmissible. A trace of a lysed suspension of microbes, introduced after filtration into a new suspension of the same species, produces there an identical lysis, and one finds then that this second suspension thereafter contains as much lytic principle as the first. One can thus carry out, having recourse on each occasion to filtration of the products of lysis, an infinite series of passages. The principle reproduces itself indefinitely. That is explained very simply by the theory of a virus which, passing the filter, would be inoculated from one lysed suspension to the next. But if one believes that the lytic principle is reproduced by the very bacteria which undergo lysis, one is forced to admit that the principle possesses, in addition to the power of causing lysis, the property of compelling bacteria to fabricate a principle identical with itself; in other words, one is driven to accept the idea that the principle, in the process of its action, automatically entails its own reproduction. The theory of autolysis implies accordingly that the sensitive bacteria already possess materials necessary for the formation of this principle, before they have been brought into contact with it. One thus comes back to the idea that the original source of the principle is to be found in normal physiology. The course of events is such as it would be if, under normal conditions, the principle already existed in the bacterium, in a very slightly active or inactive form, and if the addition of the truly lytic principle transformed this inactive form into one energetically lytic, the indefinite reproduction of this powerful lytic agent being thus assured. It may, indeed, appear strange that bacteria should have at their disposal material designed to enable an active substance to be reproduced by the mere fact of its own action. But if one reflects, this idea, though somewhat disconcerting at first, soon appears to be quite plausible. The indefinite continuation of life must, it appears, require arrangements which provide for the automatic regeneration of certain active principles; certain mechanisms, it seems, are perpetuated precisely because they, in some way, renew themselves automatically. There exist, moreover, in physiology, examples which one could think of in this connection, notably that of the agent, thrombin, responsible for the coagulation of blood, which, in acting, brings about its own reproduction. One of the constituents of thrombin, serozyme, exists in the plasma in the form of an inactive mother-substance which I have called "proserozyme." Then I found long ago that, together with its directly

coagulating action, thrombin has the power of transforming this inactive mother-substance into serozyme ready to unite, in order thereby to constitute thrombin, with the other constituent "cytozyme," which, as I showed with Delange, is of a lipoidal nature. The study of this mechanism shows then that thrombin not only provokes coagulation, but in addition, determines the very rapid production of an additional quantity of thrombin identical with itself, and it is precisely in this way that the continuity and extension of coagulation are assured. At the beginning of the process, it is the contact with some solid foreign body which represents the essential factor for the transformation of proserozyme into serozyme. But when once the contact has enabled a certain dose of thrombin to be formed, the influence of the contact is no longer indispensable, the thrombin itself producing an identical effect.

I apologise for insisting a little on these considerations. They are necessary for the comprehension of our aforementioned theory of transmissible autolysis, and they are also indispensable in enabling one to mark out the route along which experimental investigation will be able to furnish confirmation of this theory. What, in fact, is it important to demonstrate, in order that this theory may find a solid basis of facts?

If one imagines that the physiological mechanism which presides over mutations—that is to say, over the genesis, within the frame of a single species, of distinct types of microbes—represents the primary cause which has led to the appearance of the lytic principles, it is to be presumed :—

- (a) that the effects produced by the bacteriophagic principles will have some relation to the phenomena of mutation, and that,
- (b) in studying the behaviour to one another of the different types occurring in pure and perfectly normal cultures, one will find that these types manifest an antagonism to one another revealing itself by lytic phenomena, which, if not as intense, are at least closely analogous to the bacteriophagy determined by the most active and best characterised lytic principles.

I believe I have shown that experience confirms such predictions. I should not, for lack of time, be able to give an account of the whole series of investigations which I have pursued for the past ten years on this subject. I must restrict myself to the mention of those, among the results obtained, which appear to me to possess the clearest significance.

Let us take the case of a bacteriophage which is very active on *Bacillus coli*,

and which has been frequently reproduced by mixing it with suspensions of this bacillus, the microbes being then eliminated by heating to 58°, or, preferably, by filtration. I had quite early observed that, if one adds, to an extremely weak dose of this principle, an enormous quantity of *Bacillus coli*, the principle refuses to act and to reproduce itself. It disappears, and one can no longer discover it. It is as if the very weak dose of the principle scattered its influence over too great a number of the bacilli and thus could not act on them with the energy which is required to induce the reproduction of the principle and the correlated lysis. We may remark, in passing, that this result is not favourable to the virus theory; it would be difficult to suppose that a parasite would disappear, when one presents to it, in too great quantity, the bacteria which it is in the habit of attacking. However that may be, let us repeat the experiment, adding to the very rich suspension of organisms a dose of the lytic principle which, though still very small, is a little stronger than that in the preceding experiment. One finds under these conditions that the principle reproduces itself; but one observes that the principle thus obtained, which subsequently can be abundantly reproduced, by acting on moderate quantities of the colon bacilli, is no longer identical with the initial principle. It is modified: it now deserves the name of "weak principle," in the sense that, henceforward, it produces only a very incomplete lysis of cultures of *Bacillus coli*. And this modification is really qualitative and not quantitative, for this "weak" principle is still capable of acting in high dilution, just as did the original principle. But even when it is in high concentration it only produces a partial lysis, whereas the original principle clarifies the cultures very powerfully. One obtains this weak principle again, and very easily, by introducing, into a tube of bouillon which has just been given a large inoculation of *Bacillus coli*, the initial principle in a moderately large dose, such as one two-thousandth part of a cubic centimetre, and by not allowing it to act on the microbes for more than a very short time. For this purpose, immediately after the principle has been introduced into the inoculated bouillon, one mixes the latter carefully and takes a drop which one spreads upon agar. This agar tube is then kept in a vertical position in the incubator. Under these conditions numerous microbes remain caught on the surface of the agar, but the greater part of the liquid in which they were swimming, and which contains the lytic principle, runs down to the bottom of the tube. The number of spots of lysis which appear in the surface culture is thus very small. Indeed, the contact of the principle with those organisms which have become deposited on the agar having been very brief, it is logical to suppose that, among these

germs, it will be those most sensitive to the lytic principle which alone will show its effects, and will thus be able correlatively to reproduce the principle and to manifest the lysis. By transplanting one of these spots into bouillon one obtains the weak principle. One finds, then, that if the experiment is so planned that the principle acts exclusively on the most sensitive of the microbes, these regenerate a lytic agent which is so exclusively adapted to them, and preserves so faithfully the trace of its origin, that henceforward it will no longer produce any effect, except on microbes of an identical character. It spares the others, to such an extent that, when mixed with the complete culture, it only causes a partial lysis. We thus arrive at this remarkable conclusion, that the peculiar characters, the individuality of a given type of microbe, are reflected in the qualities of the principle which it is capable of elaborating.

In studying this weak principle, I made in 1922 an observation to which I attach a particular value, which I have since frequently emphasised, but the significance of which appears not to have been adequately appreciated by the partisans of the virus theory. It reveals precisely a connection between the effects of the principle and the origin of mutations. Thanks especially to the researches of your countryman Dr. Arkwright, it has been established that cultures of *Bacillus coli*, when they have been maintained for some time in the laboratory, tend to split into two well-defined types. If one uses the technique of isolation on a solid medium, one finds that the separate colonies thus obtained have not all the same appearance, and that one can classify them into two quite distinct categories. Certain colonies are convex and smooth ("smooth" type), others are flat and wrinkled ("rough" type). Transplanted into bouillon, the smooth type produces a diffuse and homogeneous turbidity, the rough type grows in floccules which form a deposit, the liquid remaining clear. By the method of isolation one can obtain the two types in pure condition. This purity, however, is not maintained. Soon, and especially so in bouillon, the smooth type tends to give rise to the rough type; on the other hand the rough type, although definitely more stable, can, nevertheless, when one keeps it for a long time, produce some individuals of the smooth type. Normally the cultures of *Bacillus coli* contain the two types in comparable proportions.

How does the weak principle behave in relation to these two races, when they have been recently obtained by isolation, that is to say, when they are still quite pure? One observes this interesting fact, that the weak principle attacks by preference, and with great vigour, the smooth type, while the

rough type, on the contrary, presents a very pronounced resistance to its action. From this fact, that the two types are unequally sensitive, a remarkable phenomenon results. I have just reminded you that the smooth type, even when carefully purified, has the power of producing some individuals of the rough type. Let us introduce into a tube of bouillon a drop of the weak principle, and then inoculate with a drop of a culture of the smooth type, and place in the incubator. This type being very sensitive to the principle, the liquid remains clear for some hours. Subsequently, however, floccules appear and soon become abundant, and then form a deposit. It is the rough type which develops; one knows that it agglutinates spontaneously. The few individuals of the rough type, which the smooth culture had a tendency to produce, have resisted the action of the weak lytic principle and soon multiply luxuriantly. As a result, the smooth culture, under the influence of the weak principle, has undergone a complete metamorphosis into a rough culture. It will be understood that, if one separates the rough type from a normal culture of *Bacillus coli*, one finds that this develops normally in presence of a dose of the weak principle far higher than that which suffices to produce lysis of the smooth type. One can easily show, moreover, that the rough type of normal provenance, that is to say, separated simply by use of the ordinary technique of isolation from the normal culture of *Bacillus coli*, and the rough type which results from the metamorphosis of the smooth type under the influence of the weak principle, are absolutely identical. In view of such results, is it not plausible to think that the weak principle, to which we give this name because it only affects one of the two types of *Bacillus coli*, but which none the less exhibits a very energetic lytic action on the smooth type, might represent only a more active form of a substance which the smooth type would normally produce, and which would have the power of making the other, rough type appear at its expense? It is indeed probable that such a substance really exists. In fact, I have observed, with Renaux, that if one filters two bouillon cultures of the same age, one of the smooth and the other of the rough type, and if one inoculates the two filtrates with the smooth strain, the rough type appears in much greater abundance and more promptly in the filtrate from the smooth than in that from the rough culture. It appears, then, that the smooth type pours into its culture medium a substance which favours its own transformation into the rough type.

Let us continue the study of the weak principle. It must be noted that the resistance of the rough type to this principle, pronounced though it be, is not absolute. In this connection the factor of concentration has a striking effect.

Concentrations of the weak principle, far superior to those which would suffice to produce lysis of the smooth strain, still spare the rough. But if, into a tube of bouillon inoculated with the smooth strain, one introduces a still much stronger dose of the weak principle, the rough organism which develops is, in the end, slightly attacked. And if one then transfers to a new tube of bouillon a strong dose of this first liquid, that is to say, not only microbes in process of lysis, but also the principle in large amount, one finds that this new mixture gradually acquires the power of attacking the rough strain. Be it said in passing, as I have pointed out in 1925, the fact that concentration plays such an important part is not favourable to the virus theory. After a series of such transfers, one finds that the principle has become adapted to the rough strain, having become capable of producing a rapid lysis of this type, even in a small dose. Thenceforward one can maintain and reproduce it by making it act on the normal purely rough type. But the principle thus adapted to the rough type has not lost its original power of causing lysis of the smooth. Henceforward it deserves accordingly the name of "strong principle," since it attacks both races, while the weak principle, with which this experiment started, caused lysis only of the smooth. The original anticoli bacteriophage, from which, at the beginning of this whole series of experiments, one separated the weak principle, contained both principles. One understands that they always co-exist in the bacteriophage which acts on *Bacillus coli*, and which is reproduced by means of the normal culture of this organism. For this culture contains the two types of organism. Several authors, in particular Bail and Asheshov, have already recognised that a single bacteriophagic liquid may contain two principles, distinguishable by the size of the spots of lysis which they cause to appear in a culture on agar. They showed the existence of a principle producing large spots and a principle producing small spots. Gratia, who continued the study of these varieties of the bacteriophage in our laboratory, was able to identify the principle adapted to the smooth strain, which I had named "weak," with the bacteriophage producing large spots, whilst the principle attacking the rough strain, which I had termed "strong" because it causes lysis of both strains, corresponds to the bacteriophage producing small spots. We thus obtain direct confirmation of the idea that the principles assume particular characteristics according to the nature of the microbial varieties by which they are respectively reproduced. Although it can be derived from the weak principle, the strong principle has acquired an individuality of its own from the fact of its being reproduced by the rough strain.

It is known that the lytic principles frequently do not sterilize completely

the cultures of the organisms sensitive to them, since certain microbes adapt themselves, succeed in multiplying, and furnish resistant strains. This phenomenon of resistance affords a precise method of distinguishing the principles, since it is highly specific. The microbe tends in some way to accommodate itself to the principle with which it has undergone contact, so as to adapt itself very strictly and exclusively to the special qualities of this latter. I have reminded you already that, under normal conditions, the rough strain is tolerant of the weak principle. But contact with the latter does not render it resistant to the strong principle. The smooth strain is susceptible to both principles, but one can adapt it, for example, to the strong principle reproduced by use of the perfectly pure rough strain. The resistant microbes obtained in this manner still manifest their original sensitiveness to the weak principle. Many examples of this kind are known.

I have frequently argued in favour of this idea, which I had put forward in 1922, of an intimate relation between the individuality of each principle and the nature of the microbial type producing it; for it seems to me an essential point in forming a judgment on the theories; and I believe that such close correlations are not favourable to the virus hypothesis. Let us note yet another difference between the two principles previously mentioned. With Renaux I have observed that the weak principle is without action on the dysentery bacillus, while the strong principle has a powerfully lytic action upon it.

It will be seen that the problem is becoming simplified, in the sense that we need no longer concern ourselves with the strong principle, for its appearance is explained. In all probability it is derived from the weak principle by the adaptation of the latter to the rough strain, and its special characters are due to the fact that this strain, which is thenceforward used to reproduce it, is not identical with the smooth strain. But, if it has been demonstrated that the weak principle can become the strong principle, is it not conceivable that the weak principle, under the action of causes still obscure, may itself be derived from a principle still weaker, too weak for its existence to be betrayed by a visible lysis, which might be precisely the normal and physiological substance responsible for the appearance of the rough at the expense of the smooth strain? We will return later to the antagonism between the types.

It is, accordingly, the weak principle which deserves the deepest study. Let us spread a drop of a smooth culture on the whole surface of an agar tube, let us wait until the excess of fluid has had time to drain to the bottom of the tube, and then introduce, at the end of a fine glass rod, a small droplet of the weak principle, which we cause to flow down along the axis of the surface of the



medium ; and let us finally place the tube in the incubator. The culture soon develops. Since, however, the middle part has been touched by the principle, a clear stripe of lysis appears in this region, bounded by the neighbouring layer of microbes, which becomes progressively thicker. In the stripe where lysis has occurred, some colonies of the rough type soon appear, which, as we know, are resistant to the weak principle. But, on the other hand, one soon observes, as I pointed out in 1922, that the layer of microbes contiguous to the clear stripe, and concentrically to it, takes on a special appearance. It becomes slightly transparent, to such an extent that the clear stripe becomes surrounded with an easily visible halo, of which the breadth may attain 2 or 3 mm. If one pricks this halo with a platinum wire, without touching the contiguous clear zone, and if one inoculates bouillon with the trace of microbial matter thus removed, it may happen that the weak principle appears in this bouillon. More often it happens that one does not find any of it, and that one obtains, without any sign of lysis, a normal culture of the smooth strain. What can be the meaning of this halo ? Working recently on a principle which I have not had the opportunity of studying, Sertik has also described similar halos surrounding the zone of lysis. Being a partisan of the virus theory, he supposes that the virus, while multiplying in the zone of lysis, liberates a kind of enzyme which dissolves the microbes, and which, being well diffusible, penetrates gradually into the neighbouring layer of microbes, which it clarifies by causing a partial lysis. I do not agree with him on this last point. So far, at least, as concerns the halos which I have observed, I think that they reveal, not a phenomenon of dissolution of the microbes, but an influence which tends to confer upon these, which are of the smooth type, the characters of the rough type. My conviction rests on the appearance of the culture. I remind you that the agar-medium in question has been inoculated with the smooth type. Then, it is important to know that, if one looks through rough and smooth cultures in the evening, transilluminating them by an artificial light, a very striking difference appears. The smooth culture refracts the light, and it appears, in front of a lamp, iridescent with the colours of the rainbow. On the contrary, the rough culture is without lustre, dull and greyish, and it is also more transparent. Now, if one examines under these conditions the culture of the smooth strain, in which the deposit of a droplet of the weak principle has caused the appearance of a clear stripe surrounded by a halo, one finds that in the region of this halo the culture of microbes, instead of being iridescent, reproduces exactly the dull, greyish and more transparent appearance of a rough culture of the same age. The identity of aspect is absolute.

Commonly the halo does not long remain visible. Frequently at the end of two days in the incubator it disappears, the culture in this region takes on again the normal appearance, the germs which compose it have not been impressed with the energy required for their definitive modification. This is why, when one takes them and inoculates them on a new medium, one often obtains a culture of the normal smooth strain. What is this diffusible substance which is responsible for the production of the halo, and which emanates from the zone of lysis? Does it differ from the lytic principle, properly so called? One must consider this possibility, since frequently the re-inoculation from the halo does not restore the principle. However, we must take account of the fact that, in the halo, the dose of the active substance which has diffused must be extremely weak, while in this region the microbes have multiplied very abundantly before being touched by it. Now we know that the principle, brought in a very weak dose into contact with too great a number of microbes, cannot be recovered again. It is, accordingly, not certain that the diffusible substance, producing the halo, is different from the principle which causes the lysis in the true sense. But we cannot exclude the possibility that the lytic liquids might contain in reality more than one active substance, just as bacteriolytic or hæmolytic sera owe their action to two substances. This question needs further investigations.

However that may be, it is important to note that the power of causing a halo to appear belongs only to the weak principle, that is to say, to the principle reproduced by the smooth strain. Now this principle is characterised by its tendency to transform the smooth strain into the rough, and one finds that the halo owes its visibility precisely to the fact that, in this region, the smooth culture takes on the aspect of the rough, as if this metamorphosis were beginning to be accomplished. One finds, moreover, that the strong principle, that is to say the principle reproduced by the intervention of the rough strain, does not produce any halo. When one deposits a droplet of the principle on an agar slope which has been inoculated with the rough strain, it could not, in any case, produce a halo, since the latter, having an appearance identical with that of a rough culture, can only appear in virtue of its contrast with that of the smooth culture. In fact, one does not see any halo round the zone of lysis when one makes the strong principle act locally on a smooth culture.

If I insist on these details, it is because the result of my most recent researches, carried out with Renaux, have just emphasised their interest. It is generally admitted that the lytic principles do not produce any perceptible effect on dead organisms. Now, in working with the weak principle, we have observed a

remarkable fact. Introduced into a suspension of *Bacillus coli* of the smooth strain, whether living or killed by heating to 60°-70°, the weak principle, even in minimal doses, causes a strong agglutination. The phenomenon, already visible after one or two hours, becomes increasingly obvious later. But the strong principle, obtained by successive reproductions on the carefully purified rough type, does not produce this effect. If one takes account of the fact that the smooth type clouds the bouillon uniformly, while the rough undergoes spontaneous agglutination, is it not curious to find that the weak principle, which brings about the metamorphosis from smooth to rough, is just the one which is endowed with the power of agglutinating the microbes of the smooth type, even when they have been killed? One knows that the lytic activity of the principle disappears with heating to about 70°. At this temperature the weak principle likewise loses its property of agglutinating the dead microbes. It is probable that the weak principle, to which the rough type is resistant, does not agglutinate the killed organisms of this strain. But this is difficult to determine, in view of the fact that these organisms undergo spontaneous agglutination.

To summarize, the whole body of the facts quoted above entitles us to consider that the effects of the lytic principles have a connexion with the phenomena of mutation.

The other question raised earlier was as follows: Do the different types of microbes belonging to a single species manifest an antagonism to one another which can be revealed by the phenomena of lysis?

The reply to this question has been greatly assisted by the important discovery of Lisbonne and Carrère. In 1922 these authors have shown that different strains of *Bacillus coli*, when one cultivates them in bouillon in a mixture with the dysentery bacillus, bring about the lysis of the latter. The filtered liquid from such a culture, added to a suspension of dysentery bacilli, causes a lysis which can be transmitted in series. These authors put forth the idea that the lytic principles are the expression of the antagonism manifested by different species of microbes to one another.

These experiments were concerned with different species, but in 1923 I was able to transfer the conception of antagonism into the framework of one and the same species, thus showing that bacteriophagy really enters the domain of normal physiology. Within the bounds of a single species different types may arise, of which some are capable of starting the transmissible lysis of certain others. The technique being very simple, I can be brief. It suffices to have recourse to the usual procedure of isolation, which allows well-separated

colonies to be obtained on agar. One transfers a certain number of these to bouillon and thus obtains the daughter-cultures A, B, C, D, etc., which one maintains for a certain time in bouillon by successive subinoculations. One finds at first that the filtrates from these different cultures do not all show the same power of starting the transmissible lysis of the dysentery bacillus. When active on this bacillus, the different principles obtained are not completely identical, since the dysentery bacillus which has become resistant to one of them may have remained sensitive to the others.

But if one tests on the different microbes of the same species, all separated from the initial culture of *Bacillus coli* with the help of the method of isolated colonies, the filtrates A, B, C, D, etc., from the cultures in bouillon of these same organisms—if, for example, one lets the filtrate from A or B act on the microbes C or D—one finds that, with certain combinations, transmissible lysis appears. Later, in 1928, I obtained with Renaux completely analogous results in the case of the staphylococcus. By submitting to the process of isolation an initial culture of which the behaviour seems in other respects perfectly normal, one can extract from it strains which are clearly antagonistic. Certain of them exercise on certain others a lytic action, which is thenceforward transmissible. One can consequently say that some behave as aggressors and that the others are receptive. And one finds, further, that this aggressiveness and this receptiveness are relative, in that they can manifest themselves in different degrees according to the qualities proper to each of the cultures. A particular germ whose secretions show themselves aggressive, that is to say lytic, for certain members of its family, can show itself receptive to the lytic influence of the secretions of different members. A particular secretion which will produce a powerful lytic action on one of the microbial strains, will act only feebly or show itself inactive towards others. Of course, it should be borne in mind that in certain cases the lysis must escape notice, because it is not sufficiently intense. Further, account must be taken of the fact that, as a general rule, the strains susceptible to a principle are capable of adapting themselves to it, and thenceforward of resisting it to such a degree that, if this adaptation is promptly effected, the period of lysis, being very brief, runs a risk of not being observed. It is highly probable, moreover, that what we call adaptation consists rather of a selection assuring the predominance of germs endowed with particular characters, including even the assumption in some cases of a slightly different morphology, as we have observed in studying the principles which are elaborated in *Bacillus coli*, and which affect certain strains of the same species.

In view of such results, one is brought to believe that the total population of a pure culture is the seat of perpetual interreactions between the individuals which compose it. The appearance of the lytic power seems, indeed, to be the index of a spontaneous differentiation, but this power itself contributes to the guidance of evolution in the culture, since it induces modifications in certain receptive types. One may suppose that it represents a regulating influence, an element of discipline for the species.

To summarize, in the species *Bacillus coli* or *Staphylococcus* which we have just considered, one can detect the existence of a spontaneous lysogenic power, provided that, having separated the different strains, one brings the most aggressive into contact with the most receptive, in such a manner as to allow the phenomenon of lysis to be sufficiently intense to show itself clearly. When the re-inoculations with the colon bacilli or staphylococci are made without having recourse to this artifice, the cultures preserve a normal aspect, and no lysis is apparent in them. There are, however, species in which the antagonism of the different strains may be so pronounced that clear spots of spontaneous lysis readily appear in their cultures. This is the case with the *Bacillus pyocyaneus*. This property is truly inherent in this microbe, and cannot be eliminated even by taking care to inoculate the medium from well-isolated colonies. Now one can demonstrate that, at the site of these clear spots, which are absolutely similar in appearance to those which are produced by the most typical bacteriophages, a conflict occurs between distinct types. Repeated re-inoculation from such spots can, in fact, furnish a new race of the organism, which, in particular by its chromogenic properties, is easily distinguished from the original strain.

To this conception of a spontaneous lysogenic power, inseparable from the physiology of microbes, the partisans of the virus have opposed the idea that the latter can live in symbiosis with the bacteria, that is to say, that parasitism may very frequently occur, without necessarily causing visible lysis. This is a purely gratuitous assumption and, in addition, improbable. In spite of careful and repeated isolations, permitting re-inoculation from well-separated colonies, one does not succeed in depriving lysogenic colon bacilli, having the most normal aspect, of their spontaneous aptitude for starting the lysis of the dysentery bacillus. It would be necessary, then, to admit that each individual microbe, which at the time of its isolation gave rise to a colony in other respects healthy, was infected by the virus. Since the principles which these colonies furnish are not completely identical, as shown particularly by the study of the aggressive and receptive strains of the staphylococcus, it would be necessary

## *Echinoderm Oogenesis.*

also to admit that the virus can diversify itself into many unequally active races. Finally, considering the weak principle studied above, which causes metamorphosis of the smooth into the rough type, would it not be very strange that a virus should have the effect of favouring a mutation which can be accomplished without it, in the most normal conditions, and which is, in reality, a physiological process?

Reviewing the whole of the experimental documents, is it not more rational to think that the virus does not exist, that the intense action of the bacteriophage represents nothing more than the pathological exaggeration of a normal function connected with mutations, and that this lysis is, in reality, as I have believed myself entitled to affirm for ten years past, since my first researches with Ciuca, a transmissible autolysis?

611.013.16 : 593.95 *Antedon*

### *Studies on Echinoderm Oogenesis.\*—I. Antedon bifida (Pennant).*

By L. A. HARVEY, M.Sc., Lecturer in Zoology, University of Edinburgh.

(Communicated by J. H. Ashworth, F.R.S.—Received October 28, 1930.)

#### *Introduction.*

The eggs of Echinoderms have provided material for a vast amount of research, much of it classical, not only on problems of hybridisation and artificial parthenogenesis, but also on the physiology of the egg. It is an extraordinary fact, however, that it has not been considered worth while to study the history of the egg during its formation and growth, with a view to arriving at a true estimate of the structures present in the mature egg and in the blastomeres derived therefrom. This statement should be qualified, in that sundry papers were published when modern cytological methods were in their infancy, but since 1906 only one serious attempt has been made to study Echinoderm oogenesis, Wilson (1926) referring all too briefly to the structures present in the eggs of Arbacia.

\*The expenses of both sections of this enquiry have been partly defrayed by grants from the Earl of Moray Endowment of the University of Edinburgh.

So much important work has been performed on the eggs, in particular of the Echinoidea, that it seemed essential that there should be some account of the origin, history and interrelationships of the bodies present in the cytoplasm of the eggs. Further, it seemed interesting to compare the oogenesis of the Pelmatozoa with that of the Eleutherozoa, and hence *Antedon bifida* and *Asterias rubens* were selected for comparison. The choice of a Pelmatozoan was strictly limited, but a wider selection of Eleutherozoa being possible *Asterias* was chosen as being easily obtainable on the shore near Edinburgh at all times of the year, and further, as belonging to the most primitive and least specialised of the sub-orders.

The majority of the work has been carried out in the Department of Zoology at the University of Edinburgh, and I am pleased to record my thanks to Prof. J. H. Ashworth, F.R.S., for his help and advice. Some time was spent in collecting material at the Marine Station at Millport, and all my living *Antedon* were sent to me from this laboratory. It is a pleasure to record my thanks to Mr. Elmhirst and the staff there for their constant and ready help.

#### *Material and Methods.*

The gonads of *Antedon* are situated in the pinnules, and hence one individual will supply an almost unlimited quantity of material. The animals were obtained from the Marine Station at Millport at all times of the year over a period of two or three years, the problem being laid aside at one time owing to press of other work. It was found that they travelled quite well over the short journey to Edinburgh, and were usually in very good condition on arrival. Endeavour was made to examine and fix such pinnules as were required soon after arrival in the laboratory, but on occasion it was necessary to keep the animals alive for a day or so in tanks in the aquarium. They do not live well, but remain in quite good condition for up to two days. The eggs then show no differences from eggs of animals freshly dredged and examined at Millport.

The pinnules were snipped off with scissors and dropped into the usual routine fixatives. They were dehydrated, embedded and cut without decalcification, the skeleton being sufficiently delicate to cause no trouble. Pinnules containing an abundance of young oocytes were comparatively scarce, those towards the distal end of the arm being smaller owing to a lesser content of oocytes rather than to smaller size of these. Except, however, in the swollen, ripe pinnules there are usually a sufficient number of young stages, particularly at the ends of the ovary, and hence the history is easily followed.

In addition to fixed material the study of living oocytes has proved exceptionally helpful, particularly with regard to the problem of the form of the Golgi bodies and of the yolk nucleus. The best results were obtained with unstained ovaries, but interesting observations were made after use of neutral red and brilliant cresyl blue. For the study of fats, Nile blue sulphate and scharlach R were used on fresh and on formalin fixed material.

It was found that osmic impregnation methods give a far truer picture of the Golgi bodies, and their relations to the other structures in the oocyte than do silver methods. This has already been discussed in relation to work on *Lumbricus* oocytes (1931) and need not be enlarged upon here save to confirm those results.

Observations of living oocytes under dark ground illumination was found to yield very interesting results. In contrast to the eggs of *Lumbricus*, where the Golgi bodies are invisible under this technique, they could be seen in many, but not all, oocytes, shining softly against the dark background of the cytoplasm. The yolk nucleus too could be seen under certain conditions which will be discussed in detail later.

#### Literature.

The only paper of note on the oogenesis of *Antedon* is that written by Chubb in 1906. This work is concerned with the relation existing between nucleolus, yolk nucleus and yolk. Methods for mitochondria or Golgi apparatus were little known at this period, and these cell elements were not studied. Chubb found that the nucleolus buds basophilic granules into the karyolymph and into the cytoplasm during the growth of the oocytes. These granules at first collect near the nucleus but later diffuse on to the surrounding cytoplasm. The mass of material so formed assumes a concavo-convex form, over one side of the nucleus, and is the yolk nucleus. As growth proceeds it migrates to the periphery of the egg and becomes spread out as a thin layer. During yolk formation the yolk nucleus loses its powers of staining and finally disappears, showing no signs of granular disintegration during the process. Neither is there any spatial relation between yolk nucleus and yolk. He therefore concludes that the yolk nucleus has no part in cell processes. The nucleolus is constituted by a continuous acidophil substance, and a deeply basophil material borne by the ground substance. During nucleolar activity the basophil material is elaborated and thrown into the karyolymph, there assuming a spherular form. The acidophil material is not extruded. On conclusion of yolk formation all nucleolar activity abruptly ceases. The acidophil content,



however, continues to be deposited on the nucleolar surface in the form of lens-shaped accumulations. From these observations Chubb deduces that the basophil material has a cytoplasmic origin—its production ceasing with cessation of cytoplasmic activity—and the acidophil portion, for similar reasons, has a chromatic origin. “The steady growth of the nucleolus is not due to the accumulation of waste material, but to an increased production of the ‘ground substance’ by the chromatin in order to cope with the ever-increasing production of waste material by the cytoplasm of the growing egg” (p. 495).

It is natural that, with the limited means at his disposal, Chubb did not find any part of the oocyte particularly concerned in yolk formation. “Yolk formation,” he says, “consists simply in the rapid and automatic conversion of the accumulated material into a form sufficiently stable to survive the period of quiescence which succeeds the completion of the egg’s growth.”

Other papers bearing on Echinoderm oogenesis will be quoted as their observations bear on my own either here or on *Asterias*.

#### OBSERVATIONS.

##### *The Mitochondria.*

The mitochondria play an obscure rôle in the oocyte. In the oogonia and youngest oocytes there is a small mitochondrial cap in the cytoplasm at one side of the nucleus (figs. 1 and 2). As the cell grows the cap increases in size, and soon its constituent granules become scattered through the cytoplasm. The granules are extremely numerous, particularly in the perinuclear zone which is formed during the spreading out of the cap. At first it is narrow and contains nearly all the mitochondria in the oocyte, but it widens rapidly and eventually extends two-thirds of the distance from the nuclear membrane to the cell surface. At about this time yolk has begun to be formed, and the clumps of yolk granules lie chiefly among the more sparsely scattered mitochondria at the periphery of the oocytes, or just on the fringe of the thicker zone. As vitellogenesis proceeds the distribution of the mitochondria becomes more even, and eventually they lie in the thin strands of cytoplasm between the yolk spheres, and are more or less obscured by them.

##### *The Golgi Apparatus and Yolk Formation.*

The Golgi bodies can be seen in living unstained eggs as colourless scales of refractive index not very much higher than that of the cytoplasm (figs. 16

and 17). In fixed preparations they are best seen after osmic impregnation, preceded or not by one of the usual fixatives—Mann, Champy, Nassonov. They are also visible occasionally in Flemming without acetic or Champy preparations stained with iron hæmatoxylin or Bensley-Cowdry. After the latter the scales are usually greenish in colour, since only rarely do they take the fuchsin. The sphere material, which is only visible in fixed preparations, in the concavity of the scale, also is stained green, intermediate in tint between the scale and the cytoplasm. This staining reaction of the Golgi bodies is interesting in connection with Parat's theory that the lepidosomes or dictyosomes of *Helix* spermatocytes, and others, are modified mitochondria, and do not represent the Golgi bodies (Parat and Gambier, 1926 ; Parat and Painlevé, 1926). This theory will be discussed in greater detail at a later stage. Iron hæmatoxylin stains the scale black or deep blue, and the sphere material grey. Silver methods were tried with little success.

Although with direct illumination the Golgi bodies appear as curved rodlets, careful examination reveals that in both fixed and living material the rodlet is the optical section of a scale-like body. Some attempt has been made to show this scale structure in one of the Golgi bodies of fig. 20, but otherwise they have been represented by single lines for convenience of drawing and reproduction. The use of dark-ground illumination on living tissue brings out the scale-like nature of the Golgi bodies more readily than other methods. By this technique no sharp outline is at any time seen, and most frequently the Golgi bodies remain totally invisible, although visible in the same oocyte by direct light (*cf. Lumbricus*, Harvey, 1931). Sometimes, however, if a prominent Golgi body be located by direct illumination, and dark ground be substituted at once, a softly shining, cup-like element is seen in place of the ring- or horse-shoe-like structure seen under previous conditions. Each Golgi body is then a scale of considerable thickness with a refractive index close to that of the ground cytoplasm, and accompanied by an amorphous patch of material visible only after fixation and staining.

Gatenby and Nath's weak osmic acid technique (Gatenby and Nath, 1926) was more successful on *Antedon* than on *Lumbricus* oocytes (Harvey, 1930). After about 10 to 20 minutes in 1 per cent. osmic acid solution the whole cytoplasm is pale brown, the yolk nucleus stands out prominently by reason of the heightening of its refractive index, and the Golgi bodies, in addition to a raised index, are also slightly darker in tint than the ground cytoplasm. Consistently with results in other material the Golgi apparatus of oögonia and young oocytes is not clearly shown under these conditions.

It will be inferred from my last sentence that the oogonia and young oocytes are not very amenable to treatment designed to demonstrate the Golgi apparatus. Even after prolonged impregnation in osmic acid the scales are frequently indistinct, and I have not seen them after any other technique. In living ovaries it is rarer to find the young cells than in fixed material, since they tend to remain attached to the wall of the ovary which is left in the pinnule, but in those that have been examined there has never been any trace of Golgi bodies until a stage when the complex vortex-like body is formed (*vide infra*). Hence the complete history of the Golgi apparatus cannot be followed on living material alone. The only differences between living and fixed material is that the scales appear slightly thicker on fixation than in the living, and the sphere material makes its appearance then. The description following may, therefore, be taken to apply to both living and fixed material except as regards the young stages and the sphere material.

In the oogonia and youngest oocytes the Golgi apparatus lies at one side of the nucleus, usually in the form of a double scale or a single U-shaped element (fig. 3). As the oocyte grows so also the Golgi apparatus enlarges, not by enlargement of individual scales, but by increase in numbers of these (fig. 4). The newly-formed scales are disposed more or less concentrically to the original ones, with their concave sides facing centrally. The sphere material thus fills the spaces between the scales and causes a general darkening of the area occupied by the apparatus. The mode of origin of the scales has not been discovered, nor whether they appear internally or cortically. Soon, however, the Golgi apparatus acquires a more or less vortex-like appearance (fig. 5). It still enlarges until it is quite complicated, and very shortly after this single scales begin to appear in the cytoplasm apart from the complex. Again it has been impossible to determine their mode of origin. The complex apparatus remains in oocytes with several single scales scattered in the cytoplasm, and the latter usually show no positional relation to the vortex. Evidence from slightly older oocytes, however, suggests that they have broken away from the vortex and wandered into the cytoplasm. I have never seen anything suggesting origin of scales *de novo*.

In slightly older oocytes the vortex-like structure begins to unwrap itself (fig. 6). There are by now several scales scattered in the cytoplasm, already mostly peripheral in distribution. The main portion of the apparatus resolves itself into a number of separate scales which begin to scatter, and after this no sign of the complex Golgi apparatus can be found in the oocyte. The scattered scales come to lie almost entirely in the peripheral cytoplasm, many

of them as shallow saucer-like elements, but others associated in twos and threes to form deep cup-like bodies (figs. 7, 8 and 9), which are very characteristic of the oocytes of *Antedon*.

Meanwhile the first yolk granules begin to appear. From the very beginning there is an intimate relationship between the newly-appearing yolk droplets and the Golgi bodies, which is maintained until the yolk is sufficient in quantity to obscure the greater number of the scales. A glance at figs. 7 to 11, 16 and 17, will convey at once what this relation is. On the concave sides of the scales, and inside the cups formed by two or more scales, appear minute granules, colourless in the living, pale green or brown after Bensley-Cowdry, black after iron hæmatoxylin. They enlarge to a certain extent, still in association with the Golgi bodies, and finally migrate away from them and form little clumps of up to 20 or 30 granules in the peripheral cytoplasm of the egg. As they enlarge they begin to stain pink, deepening to red, after Bensley-Cowdry, but otherwise show no change in colouring. Further enlargement of yolk spherules seems to take place after they have severed connection with the Golgi bodies, for no spherules more than about a third the size of the fully-formed yolk spherules were seen associated with the scales. In fig. 20 are drawn to large scale several Golgi bodies with their associated yolk droplets. It will be noticed that there is an extraordinary resemblance to some of the figures published of secretion production in various gland cells, the most striking difference being that in no case do the yolk spherules ever appear in contact with the scales, whereas the secretion droplets often arise closely applied on the strands of the Golgi network in gland cells.

The yolk content of the oocyte increases rapidly and considerably, accompanied in its earlier phases by separation of the Golgi elements of the cups. The bulk of the cell is considerably extended by reason of this increase of yolk, and at first a deep peripheral band of spherules is formed enclosing a wide space round the nucleus in which the majority of the mitochondria, and a few yolk granules and Golgi bodies, are present. Further increase of yolk fills the cytoplasm with closely-packed spherules (fig. 13). Shortly before this the Golgi bodies become very difficult to observe since they are reduced to single scales scattered among the yolk granules. The living oocytes are no longer sufficiently transparent for accurate observation, and it is only in sectioned material that they can be studied. Vitellogenesis is completed when the cytoplasm has become packed with yolk spherules to the nuclear membrane, and the Golgi apparatus and yolk undergo no further changes.

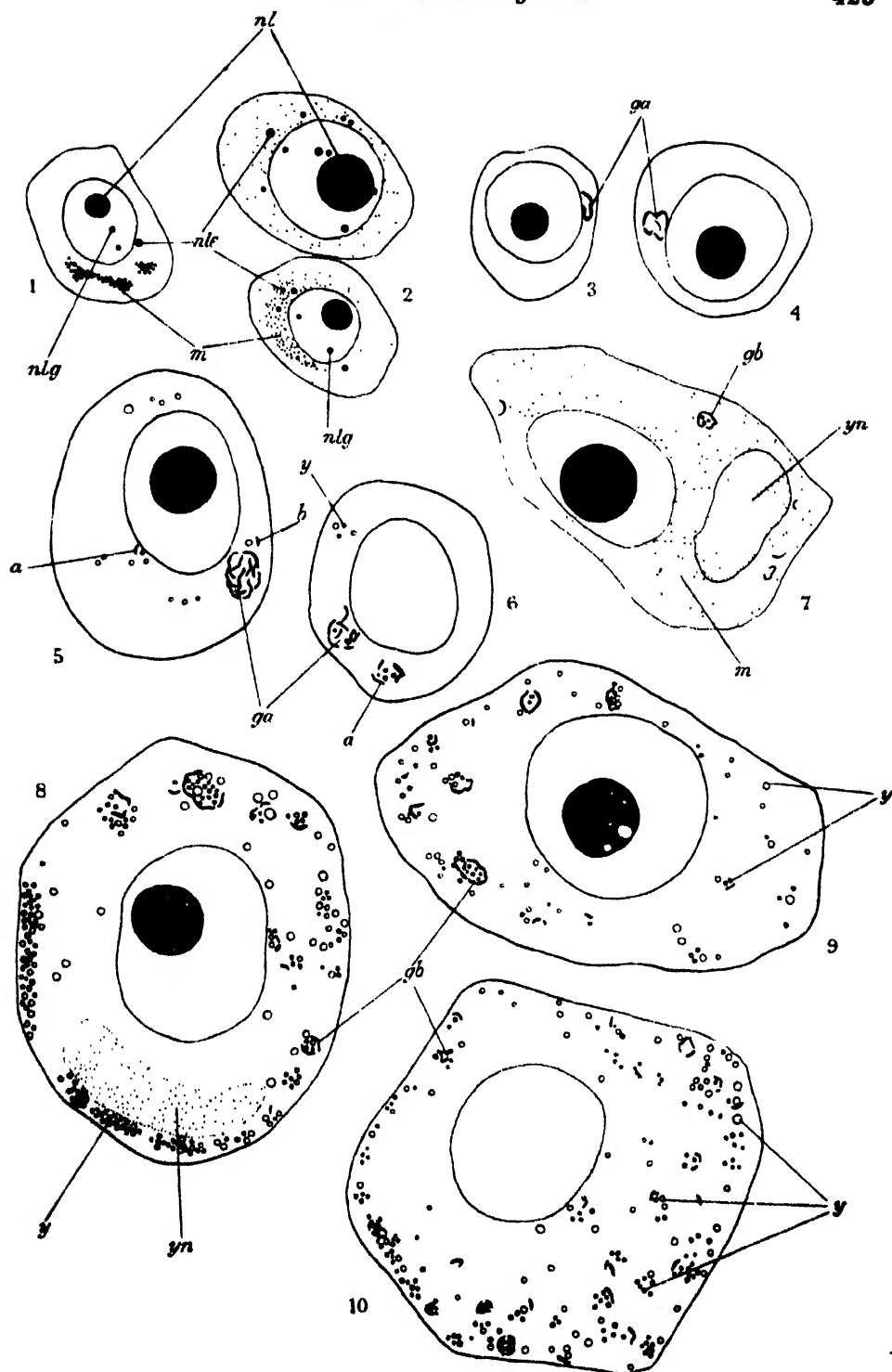
## DESCRIPTION OF FIGURES 1-10.

All figures drawn with a Zeiss 2-mm. apochr., N.A. 1.30, and compens. oc. 10 or 15.

- FIG. 1.—Very young oocyte showing cloud of granular mitochondria in the cytoplasm. A few small spherules of nucleolar material in the nucleolus, and one in the cytoplasm. Nassonov, AFATB. Mag. 1000.
- FIG. 2.—Slightly older oocytes with the mitochondria beginning to scatter, and more nucleolar spherules in both nucleus and cytoplasm. Nassonov, AFATB. Mag. 675.
- FIG. 3.—Very young oocyte to show the Golgi apparatus consisting of three scales at one side of the nucleus. Kopsch, Mag. 675.
- FIG. 4.—Older oocyte in which the Golgi apparatus has become more complex. Kopsch, Mag. 675.
- FIG. 5.—Later stage with vortex-like Golgi apparatus, one scale separated from it at *a*, another separating at *b*. A few yolk spherules already formed. Kopsch, Mag. 675.
- FIG. 6.—Showing a later phase in the unfolding of the Golgi apparatus. Some scales already separated off at *a*, with yolk granules forming among them. Kopsch, Mag. 1000.
- FIG. 7.—About one-third grown oocyte showing the yolk nucleus and one or two Golgi bodies. Also scattered mitochondria. Nassonov, AFATB. Mag. 675.
- FIG. 8.—Oocyte about half grown showing the yolk nucleus, and yolk forming at the periphery of the egg in intimate relation with the Golgi bodies. Kopsch, Mag. 675.
- FIG. 9.—Slightly smaller oocyte with a yolk nucleus, and at an earlier stage in yolk formation. Kopsch, Mag. 675.
- FIG. 10.—Another oocyte without a yolk nucleus, at a slightly later phase of vitellogenesis to show the beginning of separation of the Golgi bodies into single scattered scales. Kopsch, Mag. 675.

## EXPLANATION OF LETTERING.

AFATB, anilin fuchsin, aurantia, toluidin blue; *f.*, fat droplets; *fs.*, fatty spherules; *ga.*, Golgi apparatus; *gb.*, Golgi body; *m.*, mitochondria; *n.*, nucleus; *nl.*, nucleolus; *nl.*, nucleolar granules in the cytoplasm; *nlg.*, nucleolar granules in the nucleus; *v.*, vacuole; *y.*, yolk droplet; *yn.*, yolk nucleus.



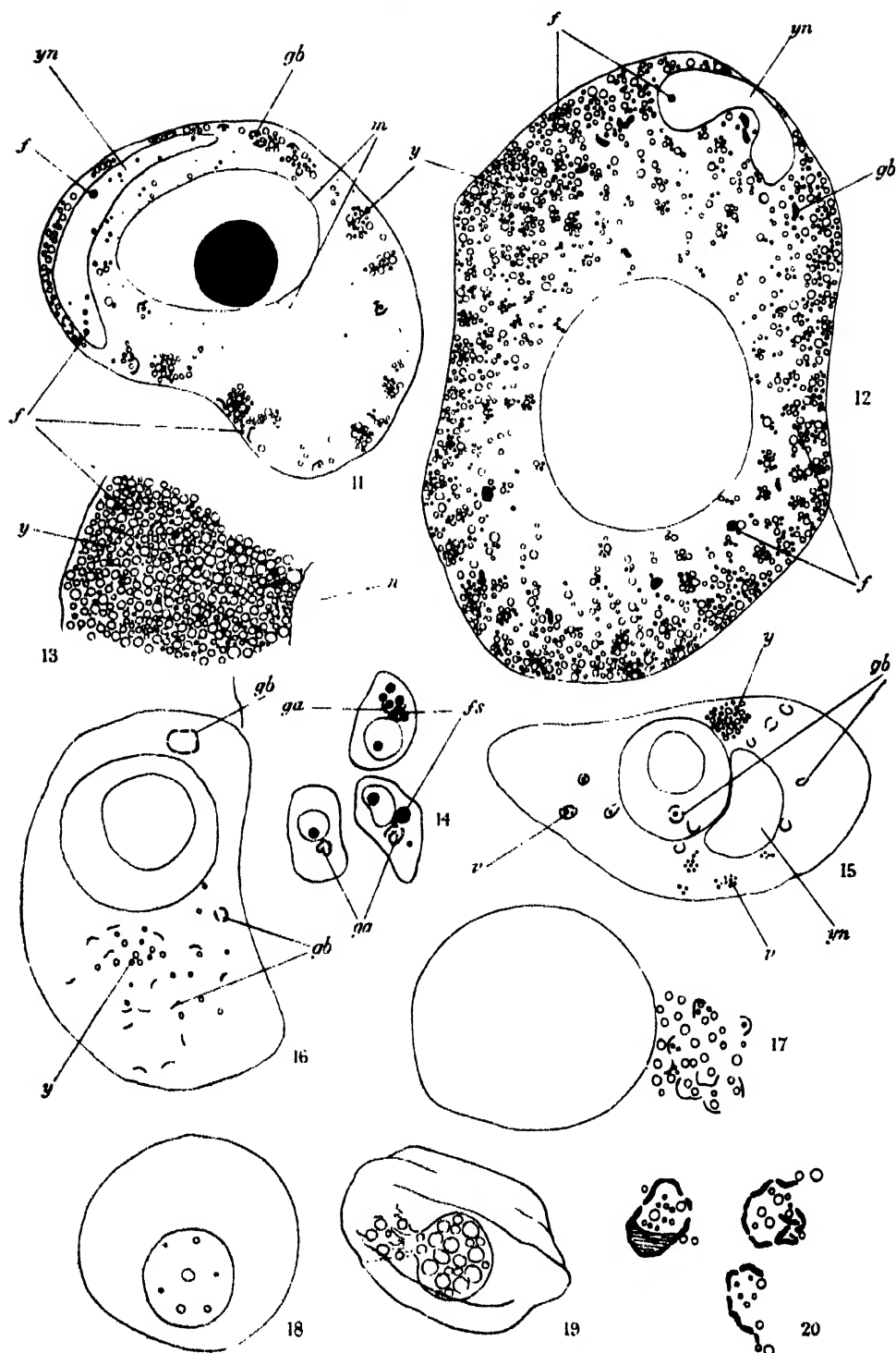
## DESCRIPTION OF FIGURES 11-20.

All figures drawn with a Zeiss 2-mm. apochr., NA. 1.30, and compens. oc. 10 or 15.

- FIG. 11.—Oocyte showing the yolk nucleus near the periphery of the cell, and containing a few large fat droplets. Smaller fat droplets are lying among the clumps of yolk granules round the outer portion of the cytoplasm. Nassonov, AFATB. Mag. 675.
- FIG. 12.—Older oocyte with yolk formation well advanced. The remnant of the yolk nucleus lies at the extreme outer edge of the cell. Many small, and a few large fat droplets among the yolk spherules, and one in the yolk nucleus. A few scattered Golgi bodies. Nassonov, AFATB. Mag. 675.
- FIG. 13.—Sector of a full-grown oocyte. The cytoplasm is packed with yolk spherules with fat droplets among them. The mitochondria and Golgi bodies are obscured by the yolk, and are not shown. Nassonov, AFATB. Mag. 657.
- FIG. 14.—Three accessory cells showing the origin of fatty spherules under the influence of the Golgi apparatus. Kopsch. Mag. 675.
- FIG. 15.—A living oocyte stained with brilliant cresyl blue. Showing the yolk nucleus and Golgi bodies, and the origin of the scattered vacuoles under the influence of the Golgi bodies. Mag. 675.
- FIG. 16.—A living oocyte showing yolk arising among the Golgi bodies. Mag. 675.
- FIG. 17.—A clump of yolk granules arising among Golgi bodies at one side of the nucleus. Living oocyte. Mag. 1000.
- FIG. 18.—The nucleus of a living oocyte. Mag. 1000.
- FIG. 19.—The same nucleus after treatment with  $2\frac{1}{2}$  per cent. hydrochloric acid showing the shrinkage of the nucleus and wrinkling of the membrane. The nucleolus has shrunk and burst at one point, some of its contents being squeezed out. Mag. 1000.
- FIG. 20.—Enlarged Golgi bodies with yolk droplets arising among them under their influence. To show the type of relation existing between them.

## EXPLANATION OF LETTERING.

AFATB., anilin fuchsin, aurantia, toluidin blue; *f.*, fat droplets; *fs.*, fatty spherules; *ga.*, Golgi apparatus; *gb.*, Golgi body; *m.*, mitochondria; *n.*, nucleus; *nl.*, nucleolus; *nlc.*, nucleolar granules in the cytoplasm; *nlq.*, nucleolar granules in the nucleus; *v.*, vacuole; *y.*, yolk droplet; *yn.*, yolk nucleus.





*The Vacuome.*

The use of neutral red and brilliant cresyl blue on this material has met with but partial success. The young oocytes in which the Golgi apparatus has not yet dispersed could not be stained, although all stages later than this showed the vacuome after about 10 minutes in stain. Brilliant cresyl blue is especially useful because of its metachromatic properties. The vacuome in this, as in other oocytes studied, is coloured deep purple, while the yolk granules are either unstained or pale sky blue, and thus complete differentiation between the two elements is effected. It has the disadvantage, however, that the colours disappear very rapidly owing to reduction of the stain by the tissues. The Golgi bodies never stain under any conditions.

The vacuoles first appear on the concave sides of the dictyosomes (fig. 15) and later migrate away from them into the cytoplasm. They are at all times very small and are not related to the yolk spherules although they arise in the same fashion. It is difficult to be certain that they are not present before the application of the stain, owing to the facts that the younger oocytes do not stain, and that there are yolk granules present in similar positions in the older cells, but probably they are secondary in origin. In the oocyte after completion of vitellogenesis small red vacuoles are scattered among the yolk and fat spherules.

The only other structure to stain in the cell was the yolk nucleus, and this only took the stain in cells damaged by the application of a solution strong enough to stain the cytoplasm. The yolk spherules sometimes took up a small quantity of stain, but as a general rule they were unstained.

*The Yolk Nucleus and Fat.*

The yolk nucleus is not present in the oogonium or young oocyte. It appears only in oocytes which have undergone a certain amount of growth, and the evidence goes to show that it is actually present in the living egg and is not an artefact. It would seem a comparatively easy problem to determine whether or no a structure is present in a living cell. The recent discussion anent the Golgi apparatus is, however, striking enough evidence to the contrary, and the yolk nucleus bids fair to be almost as elusive as the Golgi apparatus. In some ovaries of *Antedon*, and also, I should add, of *Ciona intestinalis*, the yolk nucleus is a prominent body in the cytoplasm at one side of the nucleus, in those oocytes of ages from about double the size of the oogonia to those in which the early stages of vitellogenesis have been reached. In *Antedon* it

takes the form of a transparent, homogeneous crescent, of considerable thickness in the young stage, when it forms a close cap over one side of the nucleus. Later it migrates away from the nuclear membrane towards the periphery of the oocyte and undergoes a progressive lengthening and attenuation as it moves outwards. Once at the periphery it gradually disappears as yolk formation advances. All phases of this history can be followed in the living as well as in fixed cells. On the other hand the eggs of other individuals captured at the same time and kept under identical conditions, show no sign whatever of a yolk nucleus in any of the eggs. Fixation of these ovaries in a really good fixative like Flemming without acetic or Champy fails to demonstrate it. In yet other individuals intermediate conditions prevail, in which the yolk nucleus can be detected when its position, size, and shape have been determined on other material. That is to say, no yolk nucleus is outlined, but by careful examination of particular parts of the cell a mass of cytoplasm of slightly different texture can be seen, not visibly delimited from the ground cytoplasm, in the position where the yolk nucleus is to be expected. On fixation of such an ovary and examination of stained sections, the yolk nucleus can be seen in some oocytes, but not in others. In eggs of ovaries fixed in routine histological fixatives, such as Bouin, corrosive-acetic, etc., the yolk nucleus is almost invariably seen as a deeply-stained crescentic body whose history is the same as that detailed above.

It seems justifiable therefore to assume that the yolk nucleus is present in some form or other in all the oocytes of *Antedon*, but that under absolutely natural conditions it is not optically differentiated from the ground cytoplasm. And it must be understood that the form in which it exists naturally may not necessarily be that in which it is seen under conditions of observation. Very slight deviation from natural conditions serves to render it visible in crescentic form by reason of the refraction at the interface between it and the ground cytoplasm. This interface may only be formed at this time as it is conceivable that a continuity exists between the substances of the cytoplasm and the yolk nucleus, which is very easily broken. I have not proceeded further than a few crude chemical experiments in search of the conditions governing its appearance. Briefly, the following changes do not render the yolk nucleus visible in oocytes where previously it has not been seen:—hypotonic sea-water (1 part aq. dest. to 4 parts sea-water), 5 per cent.,  $2\frac{1}{2}$  per cent., 1 per cent. hydrochloric acid in sea-water. Of these, hypotonic sea-water naturally causes considerable swelling of the cytoplasm, but it is interesting to note that the Golgi bodies remain unchanged. Further, the nuclear membrane is so inelastic

as to resist the swelling of the nucleoplasm until it is burst. The nucleolus is then ejected into the cytoplasm with some of the karyolymph and the nuclear membrane is left as a wrinkled bag. The effects of hydrochloric acid will be considered in more detail in considering the nucleolus. Addition of 5 per cent. acetic acid in sea-water, 1 per cent. osmic acid, corrosive acetic solution, or Bouin's, Zenker's, or Carnoy's fluids to the ovary causes instant appearance of the yolk nucleus. Except after osmic acid the yolk nucleus appears as a more or less fibrous coagulation effect, and after the other reagents it shows a marked shrinkage from the cytoplasm on the side away from the nucleus. After osmic acid the coagulation of both cytoplasm and yolk nucleus is so fine as to give both a clear, almost glassy, appearance.

The use of vital dyes, neutral red, brilliant cresyl blue, Nile blue sulphate, on living cells, in solutions weak enough to leave the cell more or less uninjured, has little effect on the yolk nucleus, beyond occasionally rendering it more easily seen. If the concentration of dye is raised until a marked coloration of the cytoplasm and nucleolus is obtained, the yolk nucleus is observed to take the dye more strongly than the general body of the cell, and in the case of the metachromatic brilliant cresyl blue it colours blue like the cytoplasm, in contrast to the purple of the vacuome.

It has not been possible definitely to relate the origin of the yolk nucleus with any of the structures in the oocytes. As far as can be seen it arises *de novo* in the cytoplasm at one side of the nucleus, and I can only suggest from indirect evidence that its material is derived from the nucleolus. Strong reasons will be adduced later for denying Chubb's statement (1906) that it arises from the marked nucleolar extrusions of the young oocyte. The evidence for its nucleolar origin is based entirely on the similarity of its reactions to fixatives and stains, and those of the nucleolus. As will be seen later there is a certain amount of nucleolar material in the nucleus outside the nucleolus itself, and early in the growth of the oocyte a few granules of material with the staining reactions of the nucleolus appear in the cytoplasm. These cannot be seen after a very short period, and at this time the yolk nucleolus appears, also with similar nucleolar staining reactions. Added to this, those fixing techniques which cause shrinkage of the yolk nucleus, also cause shrinkage of the nucleolus (*vide infra*). There is thus a certain amount of indirect evidence that the yolk nucleolus is derived ultimately from the nucleolus.

As to its ultimate fate there is very little to be said. It can be seen at the periphery of the oocyte during the early phases of vitellogenesis, usually with a thin layer of yolk granules between it and the surface of the egg. Later

small fragments may be seen among the yolk near the periphery, and finally it disappears completely, before yolk formation has ended.

It is not possible to leave the yolk nucleus without dealing with the history of fat formation in the egg. Fat arises at a comparatively early stage, and the first droplets always appear in the yolk nucleus, when this can be seen (figs. 11 and 12). In most cases a few droplets appear here and never increase in number beyond 20 to 30 at any stage in the growth of the egg. In the meanwhile large numbers of smaller fat droplets appear simultaneously at many points in the cytoplasm, frequently in close contact with the clumps of yolk spherules at the periphery of the egg (figs. 11 and 12). These freely scattered fat spherules are usually minute, and they arise in relation to none of the formed elements of the cytoplasm. In the well-grown egg a few larger droplets can be seen, formed probably by fusion of some of the smaller ones, but no extensive fusion takes place (fig. 12). On completion of vitellogenesis the cytoplasm is packed with yolk spherules with smaller fat droplets between them (fig. 13).

These observations on fat formation seem to show that although the first fat droplets appear in the yolk nucleus, yet this is not actively concerned in their formation. There is never any marked concentration of fat round it indicating migration of fat to or from it, and the amount of fat inside the yolk nucleus does not appear to vary greatly once the first few drops have made their appearance there. It may be that the conditions in the yolk nucleus are such that the condensation point of the fat is reached more quickly there than elsewhere in the cell, but that equilibrium is soon established as the concentration of fat rises in the body of the cell. The shrinkage of the yolk nucleus under the influence of fat solvents, and its affinity for Nile blue sulphate, among other dyes, suggest the possibility of a high fat concentration in it, and hence the more rapid saturation and condensation would be expected. I have not sufficient evidence, however, to warrant making more than a very tentative suggestion to this effect.

The fat is undoubtedly drawn from the accessory cells of the ovary. These are abortive oögonia which very early become differentiated from the definitive oöcytes by the appearance among the Golgi bodies at one side of the nucleus of comparatively large osmiophil spherules (fig. 14). The cells remain small and undergo a gradual accumulation of fat until they become converted into mere envelopes enclosing a clump of fat droplets. They are numerous, lying among the oöcytes and filling the central ovarian cavity when this is not filled by oöcytes. It is a queer paradox that fat arises in the accessory cells

under the influence of the Golgi bodies, but shows no relation whatever to them in the oocyte. It should be noted, however, that even where fat does arise in contact with the Golgi apparatus, there is no conversion of the latter into fat, until the cell has become so degenerate as to have no strictly cellular structure.

*The Nucleolus.*

In living cells the nucleolus is a colourless, translucent sphere of semi-fluid consistency. It usually contains a number of small vacuoles lying mostly at the periphery, but some occasionally occur in the inner matrix. No other differentiation has been observed. There is never any indication of budding or extrusion of material into the karyoplasm after the manner described by Chubb (1906).

In material fixed with "mitochondrial" fixatives and stained with iron hæmatoxylin, the nucleolus is deep black, the vacuoles being colourless. Ovaries stained with Bensley-Cowdry show the nucleolus either uniform reddish-orange, or pale orange with a red rim, the vacuoles being colourless. A marked differentiation is however to be seen in material fixed in corrosive acetic, Bouin, Zenker, etc. Here the core of the nucleolus is markedly acidophil, and the cortex basophil. After iron hæmatoxylin and eosin the core is pale pink, surrounded by an irregular black cortex. In this material there can occasionally be seen appearances resembling Chubb's figures of nucleolar extrusion, in which the core of the nucleolus is raised through the cortex to the surface, forming an eruption from which several droplets seem to have been ejected. It was only by accident that the real significance of this appearance was discovered. During experiments on fresh tissue to discover the nature of the yolk nucleus (*vide* p. 429) solutions of hydrochloric acid in sea-water at 5 per cent. and  $2\frac{1}{2}$  per cent. concentrations were used. After both these solutions many oocytes from about one-third to one-half grown showed the nucleolus with an almost alveolar structure, and at one side the surface raised in an eruption from which a mass of droplets had been squeezed into the nucleolymph (figs. 18 and 19). It seems, therefore, that the extrusions described by Chubb are artefacts due to the excessive contraction caused by the fixatives he used. There is no need to examine his records of further extrusion of this material into the cytoplasm, for the reasons that the greater part of them are artificial, and that Chubb was depending upon iron hæmatoxylin staining entirely, and varying only his fixing reagents. His stain is not a critical, selective stain for any structures in the cell and consequently is useless for establishing a homology between granules inside and outside the nucleus.

The droplets which he described in the cytoplasm are not therefore necessarily related to the nucleolar material at all. It must not be assumed that there is no such relation, for there are grounds for believing that an extrusion of nucleolar material in solution occurs in some eggs (Harvey, *Carcinus*, 1929), and this may be taking place in these eggs. Nothing has been seen comparable with the lace-like deposit inside the nuclear membrane of fixed *Carcinus* oocytes, but in very young oocytes the nucleus contains, besides the nucleolus, several small spherules staining as does the latter. Further, shortly after growth of the oocyte has begun, granules of similar staining material appear in the cytoplasm. These increase in number for a short while, lying scattered in the cytoplasm, and begin to dwindle and disappear in the early stages of vitellogenesis. There may, therefore, be a mild form of nucleolar extrusion taking place in the early phases of growth, but there is nothing comparable with Chubb's figures and descriptions, and the extrusions do not contribute directly to the formation of the yolk nucleus. The possibility of the ultimate origin of the yolk nucleus from the extrusions has been discussed elsewhere (*vide* p. 430).

#### Discussion.

*The form of the Golgi apparatus and its relation to the Vacuome.*—Since Parat first discovered the vacuome in animal cells there has been a prolonged dispute as to what really is the Golgi apparatus in a cell. Parat's position, which has been accepted by a number of continental workers in particular, is that the real Golgi apparatus is the vacuome, and that the classical network found in many cells after silver or osmic impregnation methods is an artefact caused by fixation in the cytoplasm around the vacuoles. In invertebrate germ-cells, and in particular in the spermatocytes of *Helix*, the dictyosomes are not strictly a part of the Golgi apparatus, but are modified mitochondria which have become associated with the vacuoles around which they lie in certain stages of spermatogenesis. There are thus two points to be considered, the relation between dictyosome and the classical Golgi apparatus, and that between Golgi apparatus and vacuome.

Nath (Nath and co-workers, 1928, 1929, 1930) claims to have demonstrated that the discrete Golgi elements of a number of invertebrate eggs, which he has examined, are in the form of vesicles, each with a rim more strongly chromophil than the interior. What this author's views are on the relation between the vesicles and, on the one hand, the dictyosome, and on the other the vacuome, I have been unable to discover. In 1930, on the grounds of Hirschler's work (Hirschler, 1928, Hirschler and Monné, 1928) and that of Gatenby (1929) on

living spermatocytes, he rejected Parat's theory of the homology of Golgi apparatus and vacuome, which he had previously inclined towards, but he still regards the Golgi elements as vacuolar in nature, and has not yet discussed the dictyosomes which have so often been described from both fixed and living material. He further claims that the Golgi vesicles are centres of fat formation, whereby they enlarge and in most cases become droplets of fatty yolk. It has been shown however (Weiner, 1930; Harvey, 1931) that in the case of the earthworm egg, at least, the so-called Golgi vesicles are nothing but droplets of weak fat and that the true Golgi apparatus exists entirely separately as small, curved, refractile rodlets, which can be seen as such in living oocytes, and on fixation appear dual in nature owing to the appearance of a small patch of weakly staining sphere material on the concave side. There is, therefore, a strong suspicion that Nath may not be dealing with a Golgi apparatus at all, but with developing fatty droplets.

In all those cases in which the Golgi apparatus has been seen in living cells it has been in the form of refractile crescentic rodlets or scales, that is as typical dictyosomes. Parat's claim that this structure is not a Golgi body ignores the work of Hirschler (1918) demonstrating the evolution of a complex net-like Golgi apparatus from the scattered dictyosomes of the blastomeres of *Limnæa*. Further evidence to the same effect is afforded by the oocytes of *Antedon*. Figs. 3 and 4 show very young oocytes in which the Golgi apparatus consists of several scales arranged concentrically and having the appearance of a network. This pseudo-network enlarges by the addition of more scales, and is at all times comparable with the classical Golgi apparatus of an inactive cell. The figs. 3 to 5 will bear comparison with the figures of inactive gland cells in many recent works. Soon, however, as the oocyte enlarges the closed vortex-like structure begins to open out, and the individual scales composing it migrate towards the periphery of the cell, where some lie singly, others associated together in twos and threes. Now these scales are exactly comparable with the dictyosomes of many other germ-cells as described by Gatenby, Ludford, Brambell, Hirschler, etc., except that they are larger than is the rule, a fact which renders the study of them more productive than that of smaller ones. Further, these scales are visible in the living oocytes and therefore are not fixation products. There seems little doubt then that the classical Golgi network is directly related to the dictyosomes of invertebrate germ cells.

Parat's postulation of the artificial nature of the net-like Golgi apparatus has gained some credence from the fact that the net cannot be seen in living cells, whereas the vacuome can be stained *in vivo*, and then occupies a position

coinciding remarkably closely with that of the Golgi apparatus, on fixation. Apart however from evidence of the fundamental secondary nature of the vacuome, the establishment of a homology between the Golgi network and the dictyosomes renders it highly improbable that the network is an artefact round a crowd of vacuoles. It must be admitted that there is probably frequently a certain amount of over impregnation whereby an unnecessary complication of the network is introduced, but that the net-like or plate-like structure is a fundamental characteristic of the Golgi apparatus of many cells cannot, I think, be doubted, the evolution of the visible dictyosome from it having once been established.

As regards the vacuome it is difficult to be certain whether it is formed under the influence of neutral red staining in these oocytes, or whether the dye merely stains preformed vacuoles. Yolk granules appear in connection with the Golgi bodies so early and the very young oocytes are so rare that considerable difficulty has been experienced in finding oocytes in which the Golgi bodies are visible and no yolk has been formed. In the one or two such oocytes seen there have been no granules or vacuoles visible in the neighbourhood of the scales, and the probability is that, as in many other tissues, the vacuoles appear as a result of the application of neutral red to the oocytes, and are therefore secondary in nature. The vacuoles remain much smaller than the yolk spherules at all times, and the latter do not as a rule stain pink after neutral red or purple after brilliant cresyl blue. In fact, after the blue dye the yolk granules if at all coloured are sky blue, in contrast to the purple of the vacuome. It is therefore evident that the two structures are entirely distinct from each other. The appearance of both on the concave sides of the scales of the Golgi apparatus affords interesting corroboration of the theory of the physical, rather than the chemical rôle of the latter, in cellular affairs.

#### *Yolk and Fat Formation.*

The oocytes of *Antedon* provide most striking figures showing the close relation between the Golgi bodies and vitellogenesis. Fig. 20 shows three groups of Golgi bodies with the associated yolk spherules. These cuplike associations of Golgi scales are very common in half-grown oocytes, together with others more open in character, and with single scales. In nearly every case, however, there are yolk droplets appearing on the concave sides of the scales, or in cases like that represented in fig. 17 a number of scattered Golgi bodies are embedded in a mass of yolk spherules in various stages of development. It cannot be doubted that yolk first appears under the influence of the



dictyosomes. Its further growth may take place, however, without their sphere of action, and hence the rôle of the Golgi apparatus in these eggs must be that of initiating a condensation, which, once started, may proceed automatically; in some such fashion crystallisation, out of a supersaturated solution, requires to be started off by provision of a foreign body as centre. It must be understood that I do not suggest that the Golgi bodies act merely in such fashion, but that the resultant small yolk spherules grow as do the crystals once formed, and hence do not necessarily require to remain in connection with the Golgi apparatus throughout their growth. By this means a comparatively small number of dictyosomes produces the enormous numbers of yolk spherules of the mature egg, filling the cytoplasm from nuclear membrane to cell surface.

As was the case in the eggs of *Carcinus*, it is not possible to ascribe more than a general synthetic rôle to the mitochondria, based entirely on outside evidence, as to the activities of these cell elements. The large numbers present in the oocytes, scattered throughout the cytoplasm, and their production early in the life of the egg, render it probable that they are concerned in yolk synthesis, although no direct evidence for this has been found.

The parts played by the nucleolus and the yolk nucleus in yolk formation will be discussed at a later stage (*vide infra*).

The appearance of the first droplets of fat in the yolk nucleus of the growing oocyte would at first sight indicate that the yolk nucleus is an organ concerned in fat production. On the other hand only a few fairly large droplets appear in it, and after this the number neither increases nor diminishes, while small droplets appear between the yolk spherules throughout the entire cytoplasm, no further relation to the yolk nucleus being apparent. It seems, therefore, that the earlier phenomenon is entirely fortuitous, and may possibly be due to an extra concentration of fat already present in the yolk nucleus, whereby the saturation point is reached at an early phase in fat formation. Thus the first condensation of fat will occur there, and after this it will proceed sufficiently rapidly over the general cytoplasm to cope with further influx from the exterior.

#### *The Yolk Nucleus.*

The yolk nucleus is a puzzling phenomenon. There are a number of animals in which there is nothing at all comparable with it in the oocytes, and yet the final result of oogenesis and vitellogenesis differs in no prominent respect from that of animals in which the yolk nucleus is present. Comparison for instance of the egg of *Carcinus* with that of *Antedon* reveals no essential difference between them. Both contain protein yolk in quantity, and fat, there

being more fat in the egg of *Carcinus* than in that of *Antedon*. Yet there is no yolk nucleus in the growing oocyte of the first, whereas it is very prominent in the oocyte of *Antedon*. An even more marked similarity exists between the eggs of *Asterias* and *Antedon*, as will be shown in Part II of this paper, and this is the more striking in that the two animals are allied, and yet the one possesses a yolk nucleus which is lacking in the other. It is tempting to draw the conclusion from these comparisons that the difference between the two types of eggs is the time factor; that in the one type a heap of raw material is produced before the egg is quite ready to proceed with vitellogenesis (assuming for the moment that the yolk nucleus is used up in this process), and in the other the material is present but is used up sufficiently rapidly never to become condensed as a visible mass.

Weiner (1925), on the grounds of observations on the eggs of *Tegenaria* and *Lithobius*, has maintained that the yolk nucleus must be regarded as "un amas de substance spécifique, élaborée par l'appareil Golgi." In addition there may be involved mitochondria and Golgi bodies, and it may include the centrosome, but all these are secondary, the only essential being the "substance spécifique." This is in essential agreement with Wilson's conclusion (1925) that on historical grounds the term must be restricted to the capsule in the centre of the pallial layer of the yolk nucleus complex as described in the egg of the spider, *Tegenaria*. This central capsule Weiner (*loc. cit.*) has shown to be formed by the Golgi apparatus, and the pallial layer consists of Golgi bodies and material elaborated by them for contribution to the capsule. Against this criterion the structure in the oocytes of *Antedon* is not necessarily a yolk nucleus at all. It is doubtful whether it is formed under the influence of the Golgi apparatus. Although there are usually a number of Golgi scales in its neighbourhood they do not seem to be active in relation to it, and there is very little evidence at all as to its mode of origin. It is, however, so closely similar to the yolk nucleus of *Ciona* which has been shown to be formed under the influence of the Golgi apparatus (Harvey, 1927), that I have little hesitation in calling it a true yolk nucleus.

Little reliance can be placed on a staining reaction as a criterion of specificity of material, yet the reactions of the yolk nucleus to stains and fixatives are strikingly similar to those shown by the nucleolus. Those fixatives which cause shrinkage and consequent eruption of the contents of the nucleolus, similarly cause shrinkage of the yolk nucleus, and after these and other fixing techniques the staining reactions are the same in both. It has already been shown that the nucleolar extrusions described by Chubb (1906) are mostly

artefacts due to nucleolar shrinkage, but there are present in the nucleus of young oocytes a number of spherules of nucleolar nature. Similar small spherules are present in the cytoplasm, of undetermined origin and fate. There is thus some evidence that nucleolar matter is passing into the cytoplasm, probably in solution, so that the actual passage of material is not visible. The marked similarity between the materials composing the yolk nucleus and the nucleolus further suggests that this material once in the cytoplasm accumulates in an area close to the nuclear membrane, as the yolk nucleus.

Opinion is divided on the question of the fate of the yolk nuclear material, some attributing to it no known function, the majority, however, being of the opinion that it is concerned in yolk formation, although little direct evidence for this has been obtained. Koch (1925) found yolk arising in vesicles in the yolk nuclei of *Chilopods*, but the majority of recent authors have found that yolk begins to form usually at the periphery of the cell, at various points, and that the yolk nucleus fragments or dissolves and disappears either during the early stages of this vitellogenesis or immediately prior to it. In practically all cases the yolk nucleus moves towards the periphery of the egg before or during its fragmentation, and, as has just been stated, it is at the cell periphery that yolk formation commences. There is, therefore, little reasonable doubt that in many eggs the yolk nuclear material is an important part of the matrix from which yolk is finally synthesised.

The appearance of fat in the yolk nucleus of *Antedon* is by no means unique (see Sokolska, 1923; Weiner, *Tegenaria*, 1925). Koch (1925) in particular records marked fat formation in the yolk nucleus of the early oocytes of *Lithobius*, followed by formation of protein yolk therein at a later stage, when the yolk nucleus has migrated to the periphery of the egg. This has not been recorded, however, for *Lithobius* by King (1924), Nath (1924), or Weiner (1925). Weiner (1925) has found fat forming in a fashion unknown in the vitelline layer on the surface of the yolk nucleus of *Tegenaria*. In other eggs in which the identification of the yolk nucleus has not been very exact, fat has been observed to arise in the area so called, which is in reality a mass of mitochondria, Golgi bodies and nucleolar extrusions. It seems therefore that the yolk nucleus often serves as a centre of fat condensation, but it is doubtful whether this is its primary function. The evidence presented from the study of *Antedon* suggests that fat appears early in it in virtue of its consistency rather than by reason of any active rôle in fat formation.

*Nucleolar Extrusions.*

The evidence for nucleolar extrusion in the oocytes of *Antedon* is not strong. The fortunate discovery of the artificial nature of the extrusions which figure so largely in Chubb's account of this oogenesis serves to point the moral that interpretations of fixed material should be subjected to rigorous scrutiny by observation of the living cells. It does not necessarily follow that all such descriptions of nucleolar extrusion are based on similar phenomena, but nevertheless examination of some of these cases may yield very interesting results. The theory of the extrusion of lumps of nucleolar material through the nuclear membrane into the cytoplasm has never been acceptable. The phenomenon has not been described adequately from living material, which is the sole criterion, and what little we know of the physics of the cell, and of surfaces, suggests that such a passage of material will take place in solution. Certain evidence corroborative of this has been obtained, but it is an extremely difficult point to prove. There is less theoretical ground for doubting nucleolar budding, but it nevertheless appears that due caution should be exercised before even this is stated with confidence. That exchange of material from nucleolus to cytoplasm occurs in many eggs is reasonably certain. The phenomenon has been reported so frequently, and sometimes in such marked form that there is no doubt of its occurrence in some guise or other. Further it may be found that a form of nucleolar extrusion goes on in most eggs, as possibly in the oocytes of *Antedon*, and that it becomes much more marked in some than in others, owing either to excessive nucleolar discharge, or to lag in absorption of the discharged material by the cytoplasm. So little attention has been paid to the fine details of extrusion that it is impossible as yet to make more than a tentative speculation as to what is going on.

*Conclusion and Summary.*

The general conclusion arrived at after due consideration of these results is that the mitochondria and Golgi apparatus are the essential active elements in the cytoplasm of the oocyte. The mitochondria of the oogonium and young oocyte are present in the form of a small cap over one side of the nucleus. This early enlarges and spreads out as scattered minute granules. They are present in large numbers in all the older oocytes, but no signs of their activity are evident. It is suggested on external evidence of mitochondrial function, which cannot be ignored, that they are probably concerned in the synthesis of yolk from raw materials provided in the cytoplasm. The Golgi apparatus

is at first a small body consisting of two or three scales at the side of the nucleus. It enlarges by production of further scales, in some manner undetermined, into a vortex-like structure, the concavities of the scales facing inwards. Eventually it begins to unfold and the scales migrate towards the periphery of the oocyte, there lying in ones, twos and threes, as scales or cup-like bodies. At this stage the Golgi bodies become visible in the living eggs, by transmitted, and sometimes by dark-ground illumination. The first small yolk droplets arise in intimate relation to the Golgi scales.

Much of the raw material for yolk formation is derived from the exterior of the oocyte, and all of it, of course, eventually, but it is probable that some of it passes into the cytoplasm only after treatment in the nucleolus, whence it is extruded into the cytoplasm in solution. In the cytoplasm this material usually collects in a crescentic area known as the yolk nucleus, and lying close to the nuclear membrane. At a slightly later period it migrates away towards the periphery of the oocyte, spreading and becoming more and more attenuated in the process. Arrived at the periphery it gradually dissolves in the cytoplasm, and is probably used in vitellogenesis.

As the yolk is synthesised it becomes condensed by the influence of the scale-like Golgi bodies, into droplet form; but once formed, the droplets can, and do, grow without further relation to the dictyosomes, probably by automatic accretion, much as a crystal grows, until an equilibrium point is reached. The large size of the single scales, and more so of the cup-like associations of two or more dictyosomes, renders the material particularly favourable for determination of this relationship between Golgi apparatus and yolk primordia.

The appearance of the first few fat droplets in the yolk nucleus is probably fortuitous, as after this the latter shows no further evidence of activity in fat formation. Minute droplets appear at points scattered throughout the cytoplasm, often among the new-formed yolk spherules. There is rarely any fusion of the fat drops, and at the end of vitellogenesis they lie scattered between the yolk spherules and are not a prominent feature of the cell.

#### BIBLIOGRAPHY.

- Bhandari, K. G., and Nath, V. (1930). 'Z. Zellf. Mikr. Anat.,' vol. 10, pp. 604-624.  
 Chubb, G. C. (1906). 'Phil. Trans.,' B, vol. 193, pp. 447-505.  
 Gardiner, M. S. (1927). 'J. Morph.,' vol. 44, pp. 217-266.  
 Gatenby, J. B. (1929). 'Proc. Roy. Soc.,' B, vol. 104, pp. 302-321.  
 Gatenby, J. B., and Nath, V. (1926). 'Quart. J. Micr. Sci.,' vol. 70, pp. 371-389.  
 Harvey, L. A. (1927). 'Proc. Roy. Soc.,' B, vol. 101, pp. 136-162.  
 Harvey, L. A. (1929). 'Trans. Roy. Soc.,' Edin., vol. 66, pp. 157-174.

- Harvey, L. A. (1931). 'Quart. J. Micr. Sci.' (*in the press*).  
 Hirschler, J. (1918). 'Arch. Mikr. Anat.,' vol. 91, pp. 140-181.  
 Hirschler, J. (1928). 'Z. Zellf. Mikr. Anat.,' vol. 7, pp. 62-82.  
 Hirschler, J., and Monné, L. (1928). *Ibid.*, pp. 201-227.  
 King, S. D. (1924). 'Sci. Proc. Roy. Dub. Soc.,' vol. 18, pp. 29-36.  
 Koch, A. (1925). 'Z. Zellf. Mikr. Anat.,' vol. 2, pp. 292-346.  
 Nath, V. (1924). 'Proc. Camb. Phil. Soc. Biol. Sci.,' vol. 1, pp. 148-157.  
 Nath, V. (1928). 'Quart. J. Micr. Sci.,' vol. 72, pp. 277-300.  
 Nath, V. (1929). 'Z. Zellf. Mikr. Anat.,' vol. 8, pp. 655-670.  
 Nath, V. (1930). 'Quart. J. Micr. Sci.,' vol. 73, pp. 477-506.  
 Nath, V., and Husain, M. T. (1928). 'Quart. J. Micr. Sci.,' vol. 72, pp. 403-418.  
 Nath, V., and Menta, D. R. (1929). 'Quart. J. Micr. Sci.,' vol. 73, pp. 7-24.  
 Nath, V., and Mohan, P. (1929). 'J. Morph.,' vol. 48, pp. 253-279.  
 Parat, M., and Gambier, E. (1926). 'C. R. Soc. Biol.,' vol. 94, pp. 748-749.  
 Parat, M., and Painlevé, J. (1926). 'C. R. Soc. Biol.,' vol. 94, pp. 745-747.  
 Sokolska, J. (1923). 'C. R. Soc. Biol.,' vol. 89, pp. 1395-1396.  
 Weiner, P. (1925). 'Arch. Russ. Anat. Hist. Embryol.,' vol. 4, pp. 153-164.  
 Weiner, P. (1930). 'Z. Mikr. Anat. Forsch.,' vol. 20, pp. 122-154.  
 Wilson, E. B. (1925). 'Amer. Nat.,' vol. 60, pp. 106-120.

611 . 013 . 16 : 593 . 95 *Asterias*.

*Studies in Echinoderm Oogenesis.*—II. *Asterias rubens*, Linné.

By L. A. HARVEY, M.Sc., Lecturer in Zoology, University of Edinburgh.

(Communicated by J. H. Ashworth, F.R.S.—Received October 28, 1930.)

*Introduction.*

The reasons which induced me to carry out this inquiry have been discussed in Part I. It is hoped by these comparative investigations to demonstrate the essential similarity between the eggs of *Antedon* and *Asterias*, in spite of apparent differences, such as, for instance, the appearance in *Antedon* oocytes of a yolk nucleus, which is lacking to the eggs of *Asterias*. It is further hoped that a wider generalisation may be foreshadowed, giving a standard against which to measure all types of vitellogenesis. It is only put forward with the idea that it may prove an alternative basis for work, to that which states that there is no single fundamental procedure of vitellogenesis, but that each egg is a law to itself. I have by no means overcome all the difficulties of observation and interpretation, and of reconciliation of divergent phenomena one with another. It will be said with justice that I have not in most cases attempted to overcome

them. To this charge I plead the chaotic state of the literature on yolk formation, often in one and the same animal (*e.g.*, the case of *Lithobius* as demonstrated by Harvey, 1929), which renders it, in my view, more important first to produce a solid mass of evidence drawn from as widely separated sources as circumstances allow, and having obtained this, to weigh other results against it. My standard not yet being of sufficient magnitude, I defer a general analysis and content myself with a tentative suggestion in the hope that other workers may be stimulated to investigate the conditions in other animals.

The work has been performed principally in the Department of Zoology of the University of Edinburgh, and I gladly take the opportunity to thank Prof. J. H. Ashworth, F.R.S., for the help and advice which he has accorded me.

#### *Material and Methods.*

*Asterias rubens* occurs on the shore at Port Seton within 10 miles of Edinburgh, and consequently a fresh supply of material was constantly available. Small individuals were obtained under rocks in the pools at all times of the year, but the large adults lie in somewhat deeper water except during the breeding period in the early summer months, when they migrate higher up the shore and are abundant on the rocks and sand. In the low spring tides of August and September the face of a big reef of rocks is exposed for many feet into the laminaria zone, and then the starfish can be obtained in enormous numbers on the seaward side. Fortnightly excursions were made to the shore throughout the year, and the animals collected were brought straight into the laboratory. Some were used at once, and others placed in aquarium tanks until required.

The usual routine fixatives were used, the ovaries being removed from the arms and cut into convenient sized pieces with a safety razor blade. In addition the living oocytes were studied carefully both stained and unstained, and the effects of various reagents such as weak osmic acid were observed in fresh material. Dark ground illumination also was used, but with comparatively poor results.

During a stay of a few weeks at the Marine Station at Millport on the Clyde, a number of starfish ovaries were examined from animals dredged in deep water off the station. It is a pleasure to express my thanks to the Director and Staff of the laboratory for the hospitality and service accorded me.

#### *Literature.*

Very little work has been carried out on the oogenesis of the Echinodermata since the introduction of modern cytological methods. The classical researches

of Boveri and Wilson on the structure of the cytoplasm were performed largely on Echinoderm eggs, but this paper is not concerned with them, in that they deal almost entirely with the egg after vitellogenesis has been completed. The same may be said of Hibbard's more recent paper (1922) on *Echinarachnius parma*. This author states that yolk is probably derived from mitochondria. She adduces no evidence, however, and does not deal with the question in any detail. Wilson (1926) summarises the structures present in the eggs of sundry Echinoids, principally *Arbacia*, and his figures agree in all important respects with those obtained in *Asterias*. He deals all too briefly, however, with the evolutionary aspect of oogenesis, being interested chiefly in the structure of the ripe egg. He does not discuss the question of yolk formation in the animals examined.

Jordan (1908, 1910) has examined the nuclei of growing oocytes of *Asterias forbesii*, *Echinaster* and *Cribrella*, and found a chromatic nucleolus in the younger stages which enlarges as the oocyte grows, and gives rise to secondary smaller nucleoli. These fragment further, and eventually the chromosomes are derived from them, but, especially in *Asterias forbesii*, a part of the material produced from the nucleolus is absorbed by the cytoplasm. This author was concerned with the relations existing between the nucleolus and chromosomes only, and consequently no attention was paid to any extruded material in the cytoplasm. The case of *Echinaster* is particularly interesting. The chromatinic nature of the nucleolus seems to have been determined on weak criteria, and may be that Jordan was here dealing with a case of marked nucleolar extrusion into the nucleoplasm at least. As *Echinaster* has a larger, more yolky egg than *Asterias*, it would be particularly interesting to determine whether there is marked extrusion or not.

The only recent direct observations on the vitellogenesis of Echinoid eggs are those of Marguerite Parat (1927), who states that the yolk is deposited in the vacuome (Golgi apparatus) which gradually stains less and less with neutral red as vitellogenesis advances, until at the end of the process there are hardly any vacuoles in the egg at all. Further, in the blastomeres of the fertilised egg the yolk is gradually resorbed and the vacuome reappears. This is interpreted as a process of dehydration of the vacuoles during yolk formation, followed by rehydration as the yolk is resorbed.



## OBSERVATIONS.

*The Mitochondria.*

The mitochondria are present in the oogonia and young oocytes, as a small, rather ill-defined cap of granules in the cytoplasm at one side of the nucleus (fig. 1). During the growth of the oocyte they increase in numbers to a great extent, the cap early fragments into its constituent granules and these become scattered throughout the cytoplasm, but show a tendency towards higher concentration in a wide zone around the nucleus. They play no visible rôle in vitellogenesis. The yolk spherules arise among them and gradually fill the egg, and at this stage the mitochondria are almost obscured, as they lie in the strands of cytoplasm between the spherules. They undergo no further changes.

*The Nucleolus.*

Little sign of nucleolar activity has been observed in the oocytes. After all fixatives the nucleolus stains uniformly except for a few small paler vacuoles mostly in the periphery. As the egg enlarges so also does the nucleolus, and its staining capacity increases, until from being a small translucent structure in the oogonium, it becomes densely stained and quite opaque. During this growth it moves from a position near the nuclear membrane to near the centre of the nucleus. There are no other spherules of similar material in the fixed nucleus, at any time. When the eggs are half grown, with a considerable quantity of yolk formed, the diffuse reticulum of the coagulated nucleus stains about the same colour as the nucleolus, and this may indicate possibly extrusion of material from the nucleolus into the karyolymph, but it is very doubtful evidence.

In living oocytes the nucleus is a clear vesicle and the nucleolus is colourless, transparent, and, except for a few refractive vacuoles, quite structureless. In many young oocytes prior to, and in the early stages of, yolk formation a few spherules of refractive material similar to that of the nucleolus are to be seen scattered in the nucleoplasm, and probably are nucleolar granules (fig. 14).

*The Golgi Apparatus and Yolk Formation.*

In its youngest stages the Golgi apparatus consists of two or three small scales each with its concave side facing towards the centre of the complex, and with a small patch of sphere material in the concavity. The complex lies close to the nuclear membrane at one side, as a small more or less spherular body of lightly stained material on whose surface are applied the scales which

are much more strongly stained. It grows by addition of more scales until in the yet very young oocyte it consists of 10 or 12 scales, each with its sphere material (figs. 2, 3 and 4). It then begins to open out, very much in the manner of the opening of a flower bud, the scales revolving outwards away from the centre of the apparatus (fig. 6). They then become scattered singly in the cytoplasm among the mitochondria, and migrate towards the periphery of the oocyte (figs. 7 and 8). During and after this process they increase greatly in numbers until they are scattered all round the peripheral cytoplasm, and are a marked feature of the egg. Once the Golgi bodies have been dispersed it is extremely rare to find them associated together in any degree. In marked contrast to the Golgi scales of *Antedon* (see Part I) they are scattered singly, and only occasionally are even two seen lying side by side. They never form cup-like associations of three or more scales. Since there is little gradation in size of the Golgi bodies, these eggs provide no evidence of their mode of origin and growth.

Sometimes during the course of the dispersal of the Golgi apparatus, but more often after this has occurred, yolk formation commences. In the sphere material which accompanies the Golgi bodies small spherules appear (figs. 5, 6, 8 and 9), and enlarge to a certain extent before separating off from them. The spherules are the developing yolk granules which therefore arise in very much the same fashion as do those of *Carcinus* (Harvey, 1929) and *Antedon* (see Part I). The relation of the Golgi apparatus to vitellogenesis is not so marked as in *Antedon*, but is manifested as it is in *Carcinus*. Rarely is more than one yolk granule seen in association with a particular scale, but the majority of the scales have a spherule on the concave side. Further, the first yolk granules almost invariably appear in the peripheral cytoplasm in the region where the scattered Golgi bodies are thickest. As in *Antedon*, the growth of the yolk droplets, once they are formed, seems to proceed without the further intervention of the Golgi bodies. They move away from the scales when quite small and enlarge to two or three times their original size. Meanwhile more small yolk droplets appear against the Golgi bodies, and so the cytoplasm rapidly becomes packed with yolk droplets (fig. 9). At first the peripheral cytoplasm only becomes filled, and later owing to the formation of yet more yolk the perinuclear zone becomes occupied from without inwards until the cell is filled from the nuclear membrane to the periphery, the Golgi apparatus and mitochondria becoming almost totally obscured in the process (fig. 10).

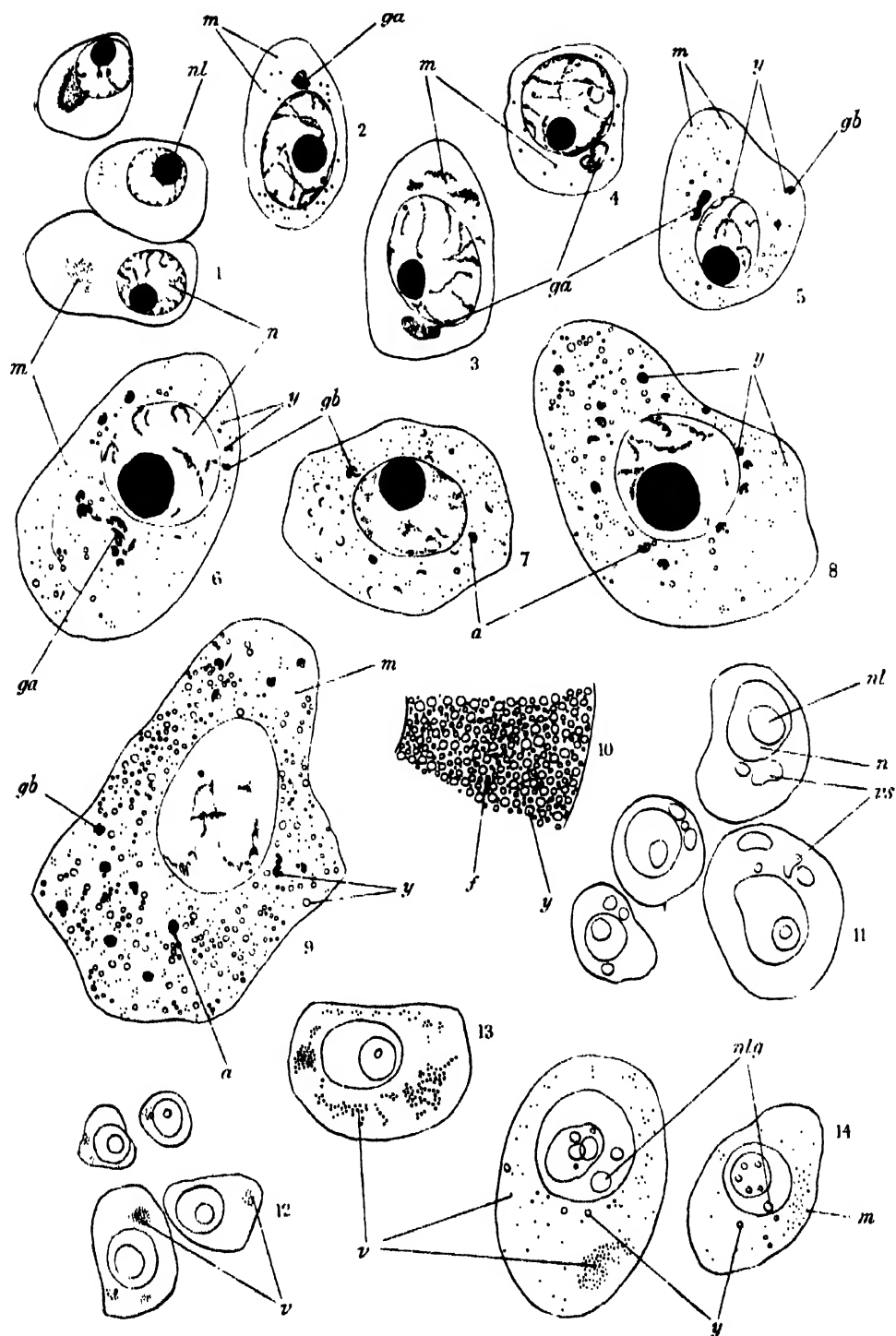
The above description has been based almost entirely on material fixed in

## DESCRIPTION OF FIGURES 1-14.

- FIG. 1.—Young oocytes showing a cloud of mitochondria at one side of the nucleus. Fwa. IH. Mag. 700.
- FIGS. 2, 3 and 4.—Young oocytes showing the complex Golgi apparatus in the early stages of growth. The mitochondria in clouds or scattered. Fwa. and urea. IH. Mag. 1000.
- FIG. 5.—Young oocyte showing precociously early dispersal of the Golgi bodies and commencement of vitellogenesis. Fwa. IH. Mag. 700.
- FIG. 6.—Oocyte showing the commencement of dispersal of the Golgi apparatus. Some scales are already scattered, the main complex is opening outwards. A few yolk granules are present, some on the concave sides of the Golgi bodies. Fwa. IH. Mag. 700.
- FIG. 7.—Oocyte with the Golgi apparatus dispersed, but yolk formation not yet commenced. Fwa. IH. Mag. 300.
- FIG. 8.—Oocyte with the Golgi apparatus dispersed and yolk spherules forming in connection with its dictyosomes. Fwa. IH. Mag. 700.
- FIG. 9.—A later stage with much yolk present, but more still appearing on the concave sides of the dictyosomes. Fwa. IH. Mag. 700.
- FIG. 10.—Sector of an older oocyte in which vitellogenesis is completed. The cytoplasm is packed with yolk spherules, with minute fat droplets between them. The mitochondria and Golgi elements are not shown, as they are obscured by the yolk. Fwa. IH. Mag. 600.
- FIG. 11.—Outline sketches of living unstained oocytes showing vesicular bodies in the cytoplasm at one side of the nucleus. Mag. 600.
- FIG. 12.—Living oocytes stained with brilliant cresyl blue in sea-water, showing the vacuome as clumps of purple vacuoles. Mag. 300.
- FIG. 13.—Older oocyte treated with brilliant cresyl blue in sea-water, showing the vacuoles more numerous and somewhat larger. Mag. 300.
- FIG. 14.—Yet older oocytes stained with neutral red in coelomic fluid. The vacuoles are scattered with small areas of greater concentration. Note the small nucleolar granules in the nuclei. Mag. 350.

## EXPLANATION OF LETTERING.

a., archoplasm; f., fat; Fwa., Flemming without acetic acid; ga., Golgi apparatus; gb., Golgi body; IH., iron hæmatoxylin; m., mitochondria; n., nucleus; nl., nucleolus; nlg., nucleolar granules in nucleus; v., vacuome; vs., vesicular bodies; y., yolk spherule.



Champy, or Flemming without acetic acid, and stained in iron hæmatoxylin. The results have been checked on material impregnated by various osmic techniques. Examination of living oocytes proved in many respects disappointing. In the older oocytes in which yolk formation had commenced no Golgi bodies could be seen. The cytoplasm appears more coarsely granular than in most eggs, which probably accounts partially for this failure. It is not surprising, however, that the Golgi bodies should be invisible, as in this they conform to the rule rather than prove exceptional. The very young oocytes corresponding to those of fixed tissues in which the Golgi apparatus has not yet been dispersed proved extremely interesting. The cytoplasm is coarsely granular and is rendered more so by the closely packed mitochondria. Nothing comparable with the scales of the fixed Golgi apparatus was seen in any oocyte, but in many a peculiar structure appeared. Lying at one side of the nucleus, and close to it, were colourless vesicular bodies, more or less ovoid or sausage-like in shape (fig. 11). The walls of the vesicles were thin, markedly refractile, and quite flexible as shown by slow changes in shape while under observation. In most oocytes there were two and sometimes more of these bodies, and in at least one case two closely apposed vesicles were seen to become confluent without involving any great change in the shape of the whole structure. Two small ovoid bodies side by side were thus replaced by one long sausage-shaped vesicle. It follows that there is very little difference in viscosity between their contents and the ground cytoplasm. In slightly older oocytes in which a little yolk has been formed sometimes one or two fairly large vesicles are still to be seen, but there are also a number of smaller ones which have become widely separated in the cytoplasm. Later still, when there are large numbers of yolk droplets in the egg, the vesicles can no longer be seen. It may be that they are obscured by the yolk, but the difference in the refractive indices of the two bodies should serve to distinguish them were the vesicles present. Probably therefore they have fragmented into minute vesicles, or have disappeared entirely, either by solution in the cytoplasm, or by a change of refractive index rendering them invisible.

In fixed preparations the vesicles are rarely found. Occasionally they are seen in chrome osmium preparations, and on staining with iron hæmatoxylin the walls are thin black lines, in optical section, enclosing an area which stains exactly as does the ground cytoplasm. They pass through the same history as the vesicles of the living oocytes, and finally can no longer be found. It appears then that they must dissolve in the cytoplasm, for the yolk granules normally stain a uniform blue or black and hence there is no possibility of confusing them with the vesicles.

The vesicles have none of the characteristics of the yolk nucleus of other eggs (*cf.* Weiner, 1925, *Tegenaria*, *Lithobius*; Koch, 1925, *Chilopoda*; Harvey, 1927, *Ciona*; Part I, *Antedon*). They are definitely vesicular with a well-developed membranous wall and watery content, whereas the yolk nucleus is characteristically a semi-solid body, frequently without a membranous wall (*vide* Harvey; Part I, *Antedon*). The cell structure which they most resemble is the Golgi apparatus, although the similarity is not marked. They pass through very similar early histories, a central accumulation at one side of the nucleus followed by enlargement and dispersal in the cytoplasm. It is a significant fact that when the vesicles have been seen in fixed preparations the scale-like Golgi bodies have not been visible. There is a possibility then that the vesicles may represent a modified form of the Golgi apparatus, and that as the latter becomes scattered in the cytoplasm it assumes scale form, and hence the vesicles can no longer be found as such.

#### *The Vacuome.*

The vacuome has not been studied in relation to the vesicular bodies described in young oocytes because it has not been found possible to demonstrate the two structures side by side. In young oocytes stained with neutral red or brilliant cresyl blue the vacuome appears in the form of scattered vacuoles of different sizes, sometimes in small clumps of up to 15 or 20 (fig. 12), more often singly or in twos and threes. In older oocytes the number of vacuoles increases considerably and they become more uniformly distributed throughout the cytoplasm, showing little or no aggregation in clumps (figs. 13 and 14). In brilliant cresyl blue they stain deep purple, whereas the yolk granules as they develop are either unstained or pale sky blue in colour. The vacuome is therefore not associated with yolk formation, being quite distinct from the yolk spherules at all times. In the fully-grown oocyte the vacuoles lie scattered at frequent intervals among the yolk and fat spherules in the cytoplasm.

There is a possibility that the vacuoles are already present in the oocyte before the application of the vital dyes, for occasionally after impregnation in osmic acid a clump of blackened granules appears in the cytoplasm of younger oocytes, similar in size and position to the vacuome. Since the Golgi bodies are invisible in the living eggs, I have not been able to determine the relations between them and the vacuoles.

*Fat.*

Fat formation commences when vitellogenesis is well advanced. Minute droplets, blackened in osmic acid, and reddened by Scharlach R, appear at many points in the cytoplasm among the yolk spherules, arising in relation to none of the formed elements of the egg. They become very numerous, and impart a greyish tint to the whole egg after osmic impregnations (fig. 10). There is never any marked fusion of the droplets and they remain minute.

*Discussion.*

A comparison of the eggs of *Asterias* and *Antedon* reveals that they are remarkably similar in final constitution, but show one marked difference in their histories. Whereas there is a prominent yolk nucleus in the younger oocytes of *Antedon*, in those of *Asterias* there is none at all. The fact that at the conclusion of vitellogenesis the eggs are almost identical in constitution suggests strongly that the yolk nucleus, as such, is not of primary importance in eggs. This evidence is supported by observations scattered in the literature on oogenesis. Those eggs which have a yolk nucleus in the younger stages finally reach a constitution which in general is the same as that in other eggs in which no yolk nucleus is present, and there is no evidence whatever correlating the presence of a yolk nucleus with any remarkable phenomena. Further there is a growing body of observation connecting the origin of the yolk nucleus with nucleolar phenomena (Weiner, 1925; Koch, 1925; Harvey, 1927). I have consequently made a tentative suggestion (*Antedon*, Part I) that its presence or absence in an egg depends entirely on the time factor; that probably there is a form of nucleolar extrusion taking place in most eggs. In some the extrusions are manifested in the cytoplasm as scattered granules staining with nucleolar reactions, and these are the eggs in which nucleolar extrusion pure and simple has been described. In others they undergo a more complicated history which results in the formation of a yolk nucleus. In yet other eggs they do not become visible at all. The reasons for these differences are that vitellogenesis commences at different times relative to the commencement of nucleolar extrusion in different eggs, and that the extruded material is used as one of the ingredients for the synthesis of yolk. The result is that in many eggs where synchronisation of the two phenomena is effected the extrusions never reach a high enough concentration in the cytoplasm to be condensed out as visible particles, whereas in other eggs extrusion has been in process so long before vitellogenesis commences, or proceeds relatively so much faster, that

the cytoplasm becomes more than saturated and the nucleolar material is thrown down either in granular form or in a particular area as the yolk nucleus. Vitellogenesis then commences and the raw material is used up, the excess going into solution as the concentration of dissolved substance diminishes. Admittedly the evidence is as yet scanty, but such a theory fits most of the facts so far brought to light, and further it explains the apparent inactivity of nucleolar extrusions and yolk nuclei in general.

The eggs of Echinoderms provide most useful evidence as to the form of the dictyosome or discrete Golgi element. Although these bodies are not visible in living oocytes of *Asterias* their form in fixed preparations is so like that of the Golgi bodies of the eggs of *Antedon* that it is justifiable to assume that in the living condition they are the same as the scales of *Antedon*. The possibility of a homology between the vesicular bodies seen in young oocytes with the Golgi apparatus is not strong enough to invalidate this statement. Now these Golgi bodies are undoubtedly scales, which in the living condition have a refractive index very close to that of the ground cytoplasm. When fixed and impregnated or stained the scale is black or dark blue, and on its concave side is an amorphous patch of material not delimited by any membrane, and staining a slightly deeper tint than the cytoplasm. At the risk of labouring the point I must insist on the scale-like nature of the Golgi elements. No question of artefact or partial impregnation is involved. They are visible in the living oocytes of *Antedon*, and also in other cells, and are most certainly not vesicular in nature. Nath's claim that the Golgi body is fundamentally a vesicle (with a chromophil rim and chromophobe centre) (Nath and co-workers, 1928, 1929, 1930) is based on insufficient evidence. He has not homologised the vesicles with, or related them in any way to, the dictyosomes described over and over again in cytological studies, and further there can be no connecting link between these vesicles and the net-like Golgi apparatus characteristic of vertebrate cells, which is the fundamental absolute to which all questions of form of the Golgi apparatus must ultimately be referred. I have been able to show in earthworm oocytes (Harvey, 1931), following Weiner (1930), that the vesicles which Nath has described as Golgi bodies are, in fact, nothing but vesicles of weak fat, and that the true Golgi apparatus exists as refractive dictyosomes scattered in the cytoplasm among the fatty droplets. It does not necessarily follow that all the vesicular Golgi bodies described by Nath and his collaborators are merely fat droplets, but it indicates the need for a morphological as well as a chemical criterion for the identity of this cell element. That the ensemble of scales or rodlets conforms to the two criteria is shown by



Hirschler in *Limnæa* (1918), where the scattered Golgi bodies of the egg and early blastomeres give rise to the complex Golgi apparatus of the tissues of the adult. This is remarkably well seen in the eggs of *Asterias* and in particular of *Antedon* (Harvey, Part I), where the Golgi apparatus of the young oocyte can hardly be distinguished from the network Golgi apparatus of inactive gland cells; yet as the oocyte grows the complex enlarges and spreads out in the cytoplasm as scattered scales.

Apart from the observation in the eggs of earthworms of the fatty vesicles called Golgi bodies by Nath, side by side with the true rod-like Golgi bodies, it is hardly to be expected that the Golgi apparatus will occupy two such fundamentally different forms as the scale or rodlet, and the vesicle, in different eggs or cells generally. The dictyosomes are the undoubted representatives of the Golgi apparatus in the majority of invertebrate eggs, and such somatic cells as have been studied. They are as undoubtedly not vesicles, but rodlets or scales with a patch of sphere material on the concave side. Acceptance of the fatty vacuoles described by Nath as Golgi elements involves admission either of a state of complete chaos in intracellular affairs, or of a diphyletic origin of the Metazoa from stocks, one with scale-like, one with vesicular Golgi bodies; and, further, intricate cases of polyphyletic origin of families, etc., since the two types of element have been described in different members of the same families. Nath's sole evidence for this is that there is a superficial resemblance between the history of the vesicles and that of the Golgi apparatus of other animals. The osmic and silver impregnation techniques are not strictly specific for the Golgi apparatus, and therefore the chemical criteria must be supported by strong morphological evidence. The only acceptable solution is then that the fatty vacuoles are nothing but fatty vacuoles, as in the oocytes of earthworms, having no connection whatever with the Golgi apparatus, and that there is one form of Golgi apparatus only in invertebrate cells, that of an ensemble of dictyosomes. The net-like Golgi apparatus of most vertebrate cells is foreshadowed in the vortex-like complex forms assumed in young eggs, of which *Antedon* provides an excellent example, and in the somatic cells of many adult forms (*vide* Hirschler, 1914, 1918).

As regards the vacuome, my own observations do not bear out those of Marguerite Parat on *Paracentrotus lividus* (1927), that yolk is deposited in the vacuome, thus leading to its gradual dehydration and diminution in the older oocytes, as vitellogenesis proceeds. On the contrary, I have not found any direct relation between the two elements, and the vacuome is present in the oldest eggs examined in the form of minute red or purple stained vacuoles

lying between the spherules of fat and yolk. They are at all times much smaller than the yolk spherules and I have never seen any granules inside them. Staining with brilliant cresyl blue demonstrates their complete independence, the vacuoles being uniform deep purple and the yolk granules unstained or pale sky blue, never with any hint of purple.

It is fast becoming a platitude that the literature on yolk formation is in a chaotic state. Anyone at all familiar with the subject knows that yolk has been derived even by modern workers from almost all conceivable sources in the oocyte—mitochondria, Golgi apparatus, yolk nucleus, nucleolus, vacuome, ground cytoplasm. It is inconceivable that such a state of affairs really exists. King (1924) first pointed out the difficulty of reconciling it with any theory of animal evolution. The storage of nutritive material in the egg is one of the most primitive of cytological phenomena, and as such it is to be expected that vitellogenesis in general will be referable to some fundamental method. It is probable that considerable variation has occurred, whereby extreme cases will differ widely. With increasing study of different types, however, it should be possible to formulate, on a broad basis, a general theory of yolk formation and trace the variants back to it. Such a theory was suggested by Hogben (1921), who was of the opinion that yolk is probably formed by the interaction of mitochondria, Golgi apparatus and nucleolus. As a result of several years of work I believe that in its widest application this will be found to fit practically all eggs. The relative importance of the three elements varies considerably, and the inherent difficulties of observation and interpretation render the details of many oogeneses obscure, so that yet more work is required before the theory can be stated with absolute confidence. There is sufficient evidence to suggest that the mean type of vitellogenesis will prove to be one in which the mitochondria are the chief synthetic organs, manufacturing yolk from raw material derived from the nucleolus as extrusions, or as yolk nucleus, and from the exterior by way of test-, follicle-, or nurse-cells. This material once synthesised is isolated out of the cytoplasmic solution by the Golgi-bodies. Fat arises by simple condensation of droplets in the cytoplasm, under the influence of none of the formed elements in the egg.

Considered in the light of this theory, the oogenesis of *Antedon* diverges very little from the hypothetical mean, while that of *Asterias* presents greater variation in that there is little sign of nucleolar activity either inside or outside the nucleus, so that probably the major part of the raw material for yolk formation is derived directly from external sources, through the test-cells which form a sparse covering over the oocyte, and through the ovarian epithelium

to which the growing oocyte is attached. In neither egg, however, is nucleolar activity manifested in the remarkable manner described for the yolky eggs of many Arthropoda and Mollusca. It would be interesting to examine the oogenesis of one of the more heavily yolked eggs of Asteroids such as *Asterina*, *Henricia*, or *Solaster* to determine whether the nucleolus is more prominently active. The results of Jordan (1908, 1910) are interesting in this respect. He found in the heavily yolked eggs of *Cribella* (*Henricia*), and *Echinaster* that the nucleolus emits particles into the nucleus in profusion. These emissions he described as chromatinic in nature, but his criteria were not absolute, and there is a possibility that he was dealing with cases of extrusion into the cytoplasm of material destined for the formation of the bulky yolk of the eggs of these species.

#### *Summary.*

The chief difference in the oogeneses of *Asterias* and *Antedon* is that whereas a yolk nucleus appears in the oocyte of the latter, there is none in that of *Asterias*.

Yolk appears in both under the influence of the Golgi apparatus, which consists of scale-like dictyosomes. In *Asterias* they are invisible in the living egg.

The mitochondria are early scattered in the cytoplasm, being present in large numbers. No sign of any activity on their part has been discovered.

Fat arises as minute droplets scattered among the yolk spherules. Neither Golgi apparatus nor mitochondria are concerned in their appearance in the cell.

There is little sign of nucleolar activity, but small spherules seen in the nucleus of young living oocytes may have been emitted from the nucleolus.

The vacuome appears as scattered vacuoles on staining living eggs with neutral red or brilliant cresyl blue. Its relation, if any, to the Golgi apparatus has not been determined. It is not concerned in yolk synthesis.

A peculiar vesicular body is described in the cytoplasm of young oocytes, and its possible relation to the Golgi apparatus discussed.

#### BIBLIOGRAPHY.

- Harvey, L. A. (1931). 'Proc. Roy. Soc.,' B, vol. 107, p. 417.  
Hibbard, H. (1922). 'J. Morph.,' vol. 36, pp. 487-492.  
Hirschler, J. (1914). 'Anat. Anzeig.,' vol. 47, pp. 289-311.  
Hugben, L. T. (1921). 'Proc. Roy. Soc.,' B, vol. 92, pp. 60-80.  
Jordan, H. E. (1908). 'Carnegie Inst. Wash. Publ.,' vol. 102, pp. 3-12 and 39-72.  
Jordan, H. E. (1910). 'Arch. Zellf.,' vol. 5, pp. 394-405.  
Parat, M. (1927). 'C. R. Soc. Biol.,' vol. 96, pp. 1360-1363.

*For further references see Part I.*

*The Relation between the Anterior Pituitary Body and the Gonads.—  
Part III.—Fractionation and Dilution of Ovary-Stimulating  
Extracts.*

By MARGARET HILL and A. S. PARKES, Foulerton Student of the Royal  
Society.

(Communicated by C. Lovatt Evans, F.R.S.—Received November 4, 1930.)

(From the Department of Physiology and Biochemistry, University College, London.)

[PLATES 33–34.]

I. *Introduction.*

The original extracts of the anterior pituitary body prepared by Evans (1924), first with saline and later with sodium hydroxide, had predominantly the effect of causing the formation of atretic corpora lutea, but it is now evident that follicular maturation and ovulation is also caused, though to a lesser extent, by these preparations. The implantation method subsequently used by Zondek and Aschheim (1927) and by Smith and Engle (1927) was found to give follicular maturation as the dominating effect to such an extent that Smith and Engle originally described this as the only effect resulting from such administration of pituitary substance. The difference in effect between the sodium hydroxide extracts and the tissue implantations suggested that two hormones were concerned in producing the two effects. It should, however, be emphasised that most workers with the sodium hydroxide extracts have neutralised the tissue mash before centrifuging to obtain the supernatant fluid for injection. In effect, therefore, the actual solvent with which the tissue is being extracted is merely saline, the only difference being that the initial treatment of the tissue leads to a much more complete extraction with the saline. The chief feature of the alkali preparations may thus be said to be more efficient extraction, and their predominantly luteinising action might be due either to the greater concentration of a single active substance, or the presence of another substance not easily obtained from implants. Conclusive experimental confirmation of the quantitative hypothesis has not been forthcoming. Courrier (1929) reported that the administration of large amounts of pituitary preparations to the cat led to the production of atretic

corpora lutea, while smaller amounts caused normal follicular maturation, but many workers have failed to obtain similar results (*cf.* Wiesner and Crew, 1930).

The evidence that two hormones are concerned is now strong. Some of the chief points are summarised below :—

- (1) Since both effects may be produced in one and the same ovary by administration of an extract, it would be necessary to suppose, on a quantitative basis, that the different follicles obtained different amounts of the substance. This, while not impossible, is improbable. Actually the dual effect of luteinisation and maturation can be observed occurring simultaneously in one follicle. This seems to dispose of the possibility that the different effects could be due to quantitative variation in the stimulus available for different follicles.
- (2) In spite of the fact that most sources of the ovary-stimulating substance give extracts producing the dual effect, sources have been described from which preparations can be obtained giving only one of the effects. Thus, Aschheim (1929) has stated that where active preparations can be prepared from the urine of non-pregnant women only follicular maturation is produced. On the other hand, Mirskaia (1930) has reported that preparations of the mouse placenta produce only luteinisation.
- (3) From sources in which both effects can be produced by crude extracts various workers have claimed to be able to dispose of one or other effects or actually to separate the two by various refinements of technique.
- (4) If a quantitative mechanism is at work, then it is necessary to suppose that the luteinisation of the follicle requires the greater amount, and, therefore, that the greater amount will be available during the luteal phase of the cycle. Yet the addition of a further supply of the substance during the luteal phase, namely, during pregnancy, results in reversal of the effect, *i.e.*, in ovulation (Engle and Mermod, 1928; Zondek and Aschheim, 1928, *a*). This experimental finding is probably the most conclusive that two hormones are concerned.

To answer the question decisively, it is obviously necessary either to (*a*) show that the administration of graduated amounts of an extract results at one end of the series in a pure luteinising effect, and at the other end in a pure follicle-maturing effect, or (*b*) to prepare two fractions which in any concentration or dilution give (i) a pure luteinising effect only, and (ii) a pure

maturation effect only. The experiments described in the present paper were designed as some approach to the problem on these lines.

## II. *Material and Technique.*

The gonad-stimulating substance or substances of urine of pregnancy are readily soluble in water and dilute alcohol, but are insoluble in concentrated alcohol (Zondek and Aschheim, 1928, *b*; Evans and Simpson, 1929, *a*). Fractional precipitation with alcohol therefore seemed to offer a possible means of purifying the extracts, or even of separating the hypothetical two substances involved. Early in our work on urine extracts, it was found that the substances were soluble in  $>70$  per cent. alcohol and insoluble in  $<80$  per cent. alcohol, and as a result the method of preparation described in Part I (Hill and Parkes, 1930) was arrived at. Aqueous, 30 per cent., and 50 per cent. alcohol extracts of the 80 per cent. alcohol insoluble material were used for the work on rabbits and ferrets there described. In the present paper are described the results of tests of (*a*) the inactive 80 per cent. alcohol soluble material, and (*b*) fractions of the active 80 per cent. alcohol insoluble material.

*Preparation of Extracts.*—The absolute alcohol and 90 per cent. alcohol soluble fractions were prepared by evaporating down the 80 per cent. soluble material, and extracting the residue successively with absolute and 90 per cent. alcohol. The dilute alcohol soluble fractions were prepared by extracting the 80 per cent. insoluble material successively with 70 per cent., 60 per cent., 50 per cent., 40 per cent., 30 per cent. and finally with water. The "total water-soluble" material was obtained by extracting the 80 per cent. insoluble material immediately with water. All extracts were prepared for injection as previously described, and concentrated so that 30 c.c. = 1,000 c.c. of original urine.

Alkali extracts of ox pituitaries were made as before, while mouse and rat pituitaries were macerated in saline for subcutaneous injection.

*Dilution of Extracts.*—To ascertain whether any quantitative explanation of the dual effect of maturation and luteinisation could be substantiated, series of dilutions of extracts were administered. Considering the concentrated extract as 1 : 1, dilutions as low as 1 : 500 were prepared and tested.

*Animals.*—With the exception of the experiments discussed in Section III, all tests were made on immature mice, 12 to 20 days old. The usual time of the onset of the first oestrus in the strain of mice used is at least 40 days. 0.5 c.c. of extract per day for 5 days was given, autopsy being performed on the sixth day. All ovaries were serially sectioned. Vaginal smearing was not performed. It is usually possible to see macroscopically whether the opening of

the vagina, if occurring, is accompanied by cornification. In particular cases the vagina was sectioned.

### III. *Dual Effect of Ovary-stimulating Preparations.*

The reactions produced by ovary-stimulating preparations of the anterior lobe and of urine of pregnancy can roughly be classified, as by Zondek and Aschheim, into (a) follicular maturation culminating in ovulation, (b) luteinisation and (c) formation of blood follicles. Two qualifications are, however, required. A mixture of reactions may be found in one follicle, and the prevalence of a given reaction varies much from species to species. Most immature follicles undergoing luteinisation exhibit various degrees of the distension typical of maturation, while many follicles undergoing what appears to be normal maturation may be found to have a small arc of luteinising tissue round the periphery of the granulosa. Similarly, most of the blood follicles in the mouse show luteinisation to some extent; in fact the distinction between blood follicles and luteinising follicles is comparatively small and, for present descriptive purposes, they may be classed together as luteinising. When the luteinising follicle has become completely solid, difficulty is experienced in distinguishing the resultant corpus luteum atreticum from a true corpus luteum formed after ovulation. Complete serial sections of the corpus luteum, showing the presence or absence of an enclosed ovum, are essential in such cases. In addition, suspected ovulation should always be confirmed by the detection of tubal ova. Confusion arising from this point may explain some of the remarkable contradictions which characterise the literature.

If two ovary-stimulating hormones are normally present in the anterior pituitary, one would expect a crude form of administration such as implantation of tissue to at least give some evidence of the dual effect. Yet Smith and Engle (1927) state that implantation of pituitary tissue gives only follicular maturation and ovulation, while on the other hand, Zondek and Aschheim (1928, c) state that maturation, luteinisation and blood follicle formation results. Conversely, Zondek and Aschheim report that urine extracts will produce normal maturation of follicles and ovulation, but Engle (1929) failed to obtain this effect. The alkali extracts of ox pituitary are variously stated to produce and not to produce ovulation in addition to general luteinisation.

Our own experience does not include implantations as carried out by Smith and Engle and by Zondek and Aschheim, but we have found that saline suspensions of mouse, rat and rabbit pituitaries, alkali extracts of ox anterior lobe and extracts of urine of pregnancy, tested on adults or immatures of one

or more species, all give a dual effect. Our results in this field are summarised in Table I. In the ferret "luteinisation" of the follicle takes the peculiar form of growth of the theca interna, degeneration of the granulosa and general cystosis of the follicle.

Table I.—Effect of various Ovary-stimulating Preparations on different Animals.

Preparation.	Maturing follicles or ovulation.	Luteinising follicles or atretic corpora.	Hæmorrhagic follicles.
Saline suspensions of pituitary tissue.....	Mouse + Rat + Rabbit +	Mouse + (3) Rat + (4) Rabbit —	Mouse — Rat — Rabbit +
NaOH extract of anterior lobe of ox pituitaries	Mouse + (1) Ferret — Rabbit +	Mouse + Ferret + Rabbit +	Mouse + Ferret + Rabbit +
Extracts of urine of pregnancy (human) ....	Mouse + (2) Ferret + Rabbit +	Mouse + Ferret + Rabbit +	Mouse + Ferret — Rabbit +

(1) See Plate 33, fig. 4.

(2) See Plate 33, fig. 5.

(3) See Plate 33, fig. 3.

(4) See Plate 33, figs. 1 and 2.

#### IV. *Dilution of Preparations.*

Dilution experiments with various ovary-stimulating preparations were carried out. The results indicate that dilution does not materially affect the nature of the ovarian reaction. The mixed effect of luteinisation and maturation is found right through the range of dilutions until a point is reached when no effect whatever is produced on the ovary. These dilution series, therefore, provide no evidence that variation in the amount of extract given produces any qualitative difference in the ovarian response. With the "total water-soluble" extracts the point at which no effect is produced on the ovary may only be reached after very considerable dilution of the preparation, dilutions as low as 1/400 still showing activity. In some of the experiments carried out in duplicate, great variation was found in the response of individual mice to the same amount of the same extract, a well-known fact which will seriously complicate the elaboration of a method of quantitative assay of ovary-stimulating activity. The results of the series of dilutions are in keeping with the findings of Evans and Simpson (1929, *b*) that once the threshold value is reached the intensity of the ovarian reaction to urine extracts is not proportional to



the amount given. Our histological finding, however, amplifies the results of Evans and Simpson, who obtained their ovarian response data mainly from the weights of the ovaries.

A further point investigated was the vaginal reaction to the various ovarian complexes produced by the dilutions of urine extracts. The two reactions observed were: (a) the typical cornification resulting from the action of oestrin; (b) proliferation of the epithelium somewhat reminiscent of the mucification which was described by Barrington (1915) in various pregnant animals, which is found during pseudo-pregnancy in the mouse, and which is apparently used by Wiesner and his co-workers (1930) as a specific indication of luteal activity. In these injected immature mice, however, this second type of vaginal reaction differs fundamentally from that found during pseudo-pregnancy. In the pseudo-pregnant mouse the mucified cells are developed from a thin vaginal epithelium, and an abundance of leucocytes are migrating through into the lumen, giving rise to the typical vaginal smear found during this phase. In the injected immature animals no leucocytes can be observed migrating through the epithelium, which is many layers thick, similar in some features to that found during pro-oestrus. A further similarity to the pro-oestrus condition is caused by signs, in some cases, that a cornified layer is beginning to form beneath the "mucified" cells. An obvious difference from pro-oestrus exists, however, in the larger size of the lining cells.

Whatever may be the interpretation placed upon the second type of vaginal reaction, the results on the immature mice receiving dilutions of urine extracts were quite inconsistent. The results were: UAP31—1/1, type *b*; 1/100, both animals cornified; 1/200, one animal cornified, one type *b*; 1/300, both cornified; 1/400, one cornified, one with cornification superseding type *b*; 1/500 one cornified, one no effect (see Plate 34). The vaginal effect, therefore, shows no relationship to the amount of extract given, and practically none to the ovarian reaction.

#### V. Fractionation of Extracts.

The results of the alcohol fractionations are given in Table II. As regards the insolubility of the substance in 80 per cent. or more concentrated alcohol, no comment is needed. The 50 per cent. alcohol preparations all produced luteinising follicles, corpora lutea atretica and blood follicles, associated with a varying but small proportion of follicles undergoing normal maturation and ovulation as diagnosed by the presence of tubal ova. The 30 per cent. alcohol fractions, on the other hand, gave a large proportion of follicles undergoing follicular maturation, luteinising follicles and atretic corpora lutea being a

minority. Certain of the preparations seemed to give pure follicular maturation, without any trace of luteinising or blood follicles. In keeping with this finding is the fact that the slight activity possessed by the residue after successive extractions with 50 per cent. and 30 per cent. was purely follicle maturing. On a quantitative theory of the different responses evoked, these results might be explained on the grounds that the first extraction\* after the 80 per cent. alcohol (50 per cent.) removed most of the activity, and the 30 per cent. extraction practically all of the remainder—in other words that the strongest extracts caused the usual multiple effects and the weaker ones predominantly or solely follicular maturation. However, the work recorded in the previous section seems to show that no such explanation is possible, and it seems likely, therefore, that a partial separation of the luteinising and maturing hormones was effected by the alcohol fractionation under the existing conditions. At best, however, the action seems to be merely that of concentrating the bulk of the luteinising hormone in the 50 per cent. alcohol, and the bulk of the follicle-maturing hormone in the 30 per cent. Whether such a fractionation method could be elaborated to separate reasonably completely the two hormones is not obvious, but since the nature of the surrounding impurities may to some extent affect the exact solubilities of the hormones, such a possibility seems remote. Further tests are, however, now being made.

Table II.—Results of Alcohol Fractionation of Urine Extracts.

Solubility.		No. of animals tested on.	Effect on test animals.
Soluble in.	Insoluble in.		
80 per cent. alcohol	—	7	No effect.
Water	80 per cent. alcohol	28	Usual multiple effect, luteinising follicles, maturing follicles, blood follicles, atretic corpora lutea, etc.
50 per cent. alcohol	80 per cent. alcohol	17	Preponderance of luteinising follicles, atretic corpora lutea and blood follicles. Little normal follicle maturation or ovulation.
30 per cent. alcohol	50 per cent. alcohol	16	Preponderance of normal follicular maturation. Some luteinisation.
Water	30 per cent. alcohol	4	Some activity; follicular maturation.

\* 70 per cent. and 60 per cent. alcohol extracts were not made as a routine.

VI. *Summary.*

1. Saline suspensions of pituitary tissue, "alkali" extracts of ox anterior lobes and extracts of urine of pregnancy all gave (a) follicular luteinisation and formation of atretic corpora lutea without ovulation, and (b) normal follicular maturation and ovulation, in one or more of the species of test animals used—mice, rats, rabbits, ferrets (Table I).

2. The relative proportions in which the two reactions occurred varied greatly with different extracts and in different animals.

3. Extensive dilution experiments with urine extracts completely failed to show that the two reactions could be ascribed to the activity of one stimulating hormone evoking different responses in different concentrations.

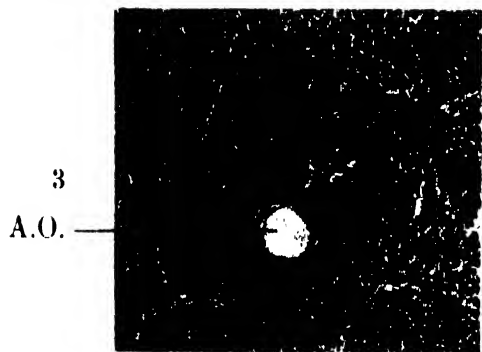
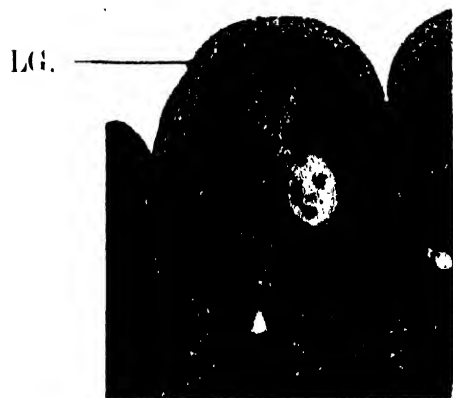
4. Alcohol fractionation of urine extracts gave some indication of a partial separation of luteinising and maturing activity. The 50 per cent. alcohol soluble material was predominantly luteinising, while the reaction to the 30 per cent. alcohol soluble fraction was mainly follicular maturation (Table II). It does not seem possible to explain this result on a quantitative basis (see (3) above).

Our thanks are again due to Prof. C. Lovatt Evans, F.R.S., and Prof. J. P. Hill, F.R.S., for facilitation of the work. To Prof. F. J. Browne we are indebted for arranging the collection of urine of pregnancy.

The expenses of the research were defrayed from a grant to A. S. P. from the Medical Research Council.

## REFERENCES.

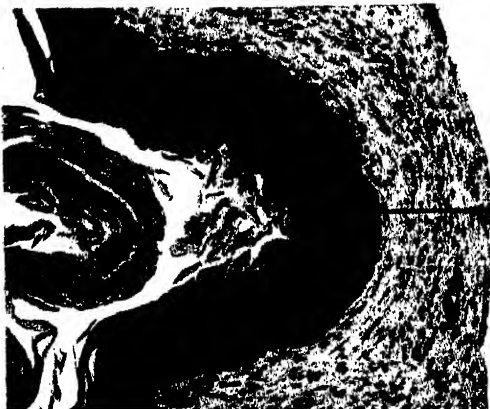
- Aschheim (1929). 'Z. Geburts. Gynak.,' vol. 94, p. 203.  
 Barrington (1915). 'J. Anat. Physiol.,' vol. 50, p. 30.  
 Courier and Kehl (1929). 'C. R. Soc. Biol.,' vol. 100, p. 711.  
 Engle (1929). 'J. Amer. Med. Assoc.,' vol. 93, p. 276.  
 Engle and Mermod (1928). 'Amer. J. Physiol.,' vol. 85, p. 518.  
 Evans (1924). "Harvey Lectures," p. 212.  
 Evans and Simpson (1929a). 'Proc. Soc. Exp. Biol., Med.,' vol. 26, p. 597.  
 — (1929b). 'Amer. J. Physiol.,' vol. 89, p. 381.  
 Hill and Parkes (1930). 'Proc. Roy. Soc.,' B, vol. 107, p. 30.  
 Mirakia (1930). 'Proc. Roy. Soc. Edin.,' vol. 50, p. 104.  
 Smith and Engle (1927). 'Amer. J. Anat.,' vol. 40, p. 159.  
 Wiesner and Crew (1930). 'Proc. Roy. Soc. Edin.,' vol. 50, p. 79.  
 Zondek and Aschheim (1927). 'Arch. Gyn.,' vol. 130, p. 1.  
 — (1928, a). 'Endokrinologie,' vol. 1, p. 10.  
 — (1928, b). 'Klin. Wschr.,' vol. 7, p. 831.  
 — (1928, c). 'Klin. Wschr.,' vol. 7, p. 1401.



1  
T.b.



C.E.C



DESCRIPTION OF PLATES.

*A.o.*, Atretic ovum. *C.E.C.*, Cornified epithelial cells. *L.G.*, Luteinized follicular granulosa. *T.b.*, Type *b* epithelial cells. *V.E.*, Vaginal epithelium.

PLATE 33.

- FIG. 1.—Atretic corpus luteum in adult rat injected subcutaneously for 4 days with saline suspension of one rat pituitary.  $\times 55$ .  
 FIG. 2.—Luteinising follicle in the same rat.  $\times 55$ . Both corpora lutea atretica and luteinising follicles were common in rats so treated, as compared with their comparative rarity in normal rats.  
 FIG. 3.—Atretic corpus luteum in immature mouse injected subcutaneously for 5 days with saline suspension of one mouse pituitary.  $\times 150$ .  
 FIG. 4.—Tubal ovum in immature mouse receiving 1/50 dilution of alkali extract (HAP3) of ox anterior lobe.  $\times 150$ .  
 FIG. 5.—Tubal ovum in immature mouse receiving  $\frac{1}{2}$  dilution of urine extract (UAP25).  $\times 150$ .

PLATE 34.

$\times 200$ .

Effect on the vagina of the immature mouse of ovarian stimulation produced by dilutions of urine extract (UAP31, see p. 460). The illustrations show the complete lack of correspondence between the ovarian and vaginal reactions. As regards cornification of the vagina, the extract may be considered to have been oestrin-free and in any case the distribution of cornification in the dilution series could not be accounted for by the presence of oestrin.

- FIG. 1.—Immature mouse receiving strong extract (1/1). Type *b*, vagina. Ovarian reaction: atretic corpora lutea, blood follicles.  
 FIG. 2.—Immature mouse receiving dilution 1/100. Cornified vagina. Ovarian reaction: atretic corpora lutea, luteinising follicles.  
 FIG. 3.—Immature mouse receiving dilution 1/200. Type *b*, vagina. Ovarian reaction slight, some follicular growth.  
 FIG. 4.—Immature mouse receiving dilution 1/300. Cornified vagina. Ovarian reaction slight, few atretic follicles.  
 FIG. 5.—Immature mouse receiving dilution 1/400. Type *b*, vagina. Ovarian reaction: atretic corpora lutea.

*The photomicrographs are the work of Mr. F. J. Pittock.*

*The Pupillary Mechanism of the Teleostean Fish Uranoscopus scaber.*

By JOHN Z. YOUNG, B.A., Christopher Welch Scholar, Senior Demy of Magdalen College (Department of Zoology and Comparative Anatomy, Oxford).

(Communicated by E. S. Goodrich, F.R.S.—Received November 19, 1930.)

INTRODUCTION.

The old opinion that the pupil of teleostean fish never varies in size was disproved by the work of Brown-Séquard (1847), who showed that in certain species (eels, flat fish) there was a very considerable light reflex. Steinach (1890 and 1892) showed that there were in fact changes in the size of the pupil in all fish, although in many cases the variations were very slight and only detectable by careful measurement. Steinach also showed that in Teleosts and Amphibia the incidence of a bright light caused narrowing of the pupil even after the eye had been removed from the head, and he concluded that in these animals the movements of the iris were produced by the direct effect of the incidence of the light on the pigmented muscle cells of the sphincter.

This opinion has persisted to the present day (Kuntz, 1929), in spite of the discovery by Magnus (1899) of the fact that the curve of the pupil reaction in respect to spectral light resembles the absorption curve of the visual purple of the retina and not that of the pigment in the iris. Magnus therefore concluded that some intra-ocular nervous reflex was involved in closure of the pupil. However, Langley and Orbeli (1910) showed that in the frog stimulation of the sympathetic chain caused dilatation of the pupil, so that in the Amphibia at any rate there is certainly an extra-ocular nervous mechanism responsible for changes in the size of the pupil, in addition to an intra-ocular mechanism (whether nervous or not), responsible for the closure of the pupil of the isolated eye. Apparently no effort has been made to discover whether there is an extra-ocular mechanism in fish.

The structure of the iris of Teleosts was shown by Grynfeldt (1910) to be very similar to that of mammals. He found a circular sphincter muscle, composed of smooth fibres, and various radial dilatator elements.

In the course of a study of the autonomic system of *Uranoscopus scaber* it was found that the pupil narrowed when the sympathetic chain was stimulated, and further investigation has shown that there is in fact a fully developed

nervous mechanism for the control of the pupil, differing in a most interesting manner from the mechanism found in Tetrapods.

*Uranoscopus scaber* (the "Stare Gazer") is a rather aberrant Teleost which lives buried in the sand, only the eyes protruding above the surface. The food consists of gobies, small crustacea, etc., which are attracted by a small strip of vascular tissue which is attached to the lower jaw and can be moved rapidly in and out to act as a lure. The mouth is very large and is directed upwards; the prey is swallowed whole. The eyes are on the top of the head and can be protruded or rapidly withdrawn into sockets.

*Uranoscopus* is quite common in the Mediterranean, and it lends itself readily to experiment, except that its time of survival in the aquarium is limited to about three weeks. It is especially suitable for a study of the pupillary mechanism, not only on account of the very marked light reflex, but also because it lies still on the bottom of a tank, allowing the eyes to be closely watched.\*

#### THE LIGHT REFLEX OF URANOSCOPUS.

##### 1. Method.

Exact measurements of the changes in the size of the pupil are not easy in a fish (see Steinach, 1892, and Magnus, 1899) and have not been attempted in the present investigation. The fish were kept in a glass-sided tank in a dark room. On the bottom of the tank was enough sand for the fish to bury themselves, leaving only the eyes visible. An electric lamp hung above the surface of the water enabled the fish to be brightly illuminated from above; for some experiments they were confined in smaller glass vessels, near to the sides of the tank, so that a torch could be shone into one or both eyes.

##### 2. Normal Light Reflex.

If a *Uranoscopus* was left in the tank and the room diffusely lit by daylight, then both the pupils were seen to be wide open. If the animal was then strongly illuminated from above by means of the electric light there were no apparent changes in the size of the pupil (exact measurement would probably

\* The investigation was carried out while in occupation of the Oxford Table at the Naples Zoological Station and during tenure of the Christopher Welch Scholarship and of a Senior Demyship from Magdalen College, Oxford. I wish to thank the respective bodies for the grant of these facilities and also Professor Dohrn and the entire staff of the Zoological Station for their always willing assistance. I am especially indebted to Professor E. Sereni for constant help and advice during the work and to Dr. M. H. MacKeith and Professor E. S. Goodrich for reading the MS.



show some change). If, however, the fish was left for some time in total darkness and then brightly illuminated, both pupils were seen to narrow during the ensuing 5 seconds until they were almost completely closed. After a further short interval they began to re-open, and after a few minutes became nearly as wide as they were previous to the illumination.

In order for the light reflex to be manifested it was found necessary to leave the animal in the dark for more than a certain minimum period (about 15 minutes). That is to say that if the light was turned on after only 10 minutes of darkness no closure followed. If the animal was left in the dark for a period rather longer than 15 minutes then the closure on illumination was only partial and the re-opening took place after an interval roughly inversely proportional to the time which had been passed in the dark. This is demonstrated by the following experiment in which an animal was left for gradually decreasing periods in the darkness, with intervening periods of 3 minutes' illumination. The times of closing and re-opening were measured with a stop-watch after each illumination.

Time of darkness.	Closure when illuminated.	Re-opening.
Minutes		
60	Complete	Begins after 1 minute
40	Complete	" 1 minute
30	Partial	" 20 seconds
15	Slight	" 5 seconds
1	None	—

This and similar experiments show that the eyes of these fish gradually become adapted to the darkness, the process reaching its maximum after about 40 minutes.

### 3. *Consensual Reflex.*

Experiments were made to determine whether the light reflex operates separately in the two eyes or if there is also a contra-lateral effect. If a torch was shone into one eye only, until its pupil had become narrow, and was then moved round to illuminate the other eye, it was found that the latter was still widely open and that it then proceeded to close under the influence of the light. This agrees with the experiments of Steinach (1890), who found no consensual reflex in eels. Further experiments, however, showed that it is possible for light entering at one eye to cause narrowing of the opposite pupil.

In two experiments the skull was opened dorsally and one of the optic nerves cut completely through. The wound was then closed and the animal

returned to the tank. Immediately after the operation the pupil of the eye whose nerve had been cut remained wide on illumination, although that of the normal eye was narrowed; but after 3 hours in one case and 20 hours in the other, the pupil on the operated side also narrowed on illumination. At first the narrowing was much less than on the normal side, but later it became almost, though never quite, as complete as the control. It seemed that the latent period after illumination and before closing was longer on the operated side (5 to 10 seconds) than on the normal side (1 to 5 seconds). The re-opening began sooner and was more rapid on the operated side than on the normal side.

In one animal in which both optic nerves were cut, the light reflex was permanently abolished on both sides, so that the direct effect of light on the iris seems to be of little importance, relative to the nervous mechanism, in regulating the size of the pupil, although it is well known (Steinach, 1892) that if the eye of fish or amphibian is removed from the head the sphincter iridis will still contract when strongly illuminated.

The above experiments show that, at least under abnormal conditions, it is possible, even in an animal with a complete decussation, for light entering at one eye to cause narrowing of the opposite pupil, although, as Steinach pointed out, a true consensual reflex is found only in those mammals with incomplete decussation of the fibres of the optic nerve.

#### MECHANISM OF THE LIGHT REFLEX.

Since the experiments of Steinach (1892) it has always been supposed that the changes in the size of the pupil of fish were the result of the direct incidence of light upon the pigmented sphincter muscles. It has been shown above that this is not the usual mechanism since, after cutting both optic nerves, the reflex closure of the pupil no longer takes place. Further investigation has shown that the regulation of the size of the pupil, here as in other vertebrates, is a nervous function.

It has long been known that in mammals the m. sphincter iridis receives its motor innervation from the oculomotor nerve via the radix brevis and ciliary ganglion, while the dilatator receives motor fibres from the sympathetic system via the superior cervical ganglion, carotid plexus and ciliary nerves.

#### 1. *Methods.*

The methods used have been electrical stimulation, experimental transection of the nerves of the living animal, and the injection and local application to

the iris of drugs and poisons. The nicotine method of Langley has also been used, with interesting results.

No attempt was made to stimulate the nerves of an animal with intact circulation, since many of the nerves lie dorsally to the heart and could only be reached with difficulty. The method adopted was to cut away the heart, the whole of the lower jaw and operculum and the gut; the sympathetic chains were then exposed for the whole of their length and by opening the skull ventrally the cranial nerves were stimulated. In such a preparation the iris muscles continue to respond for upwards of 1 hour.

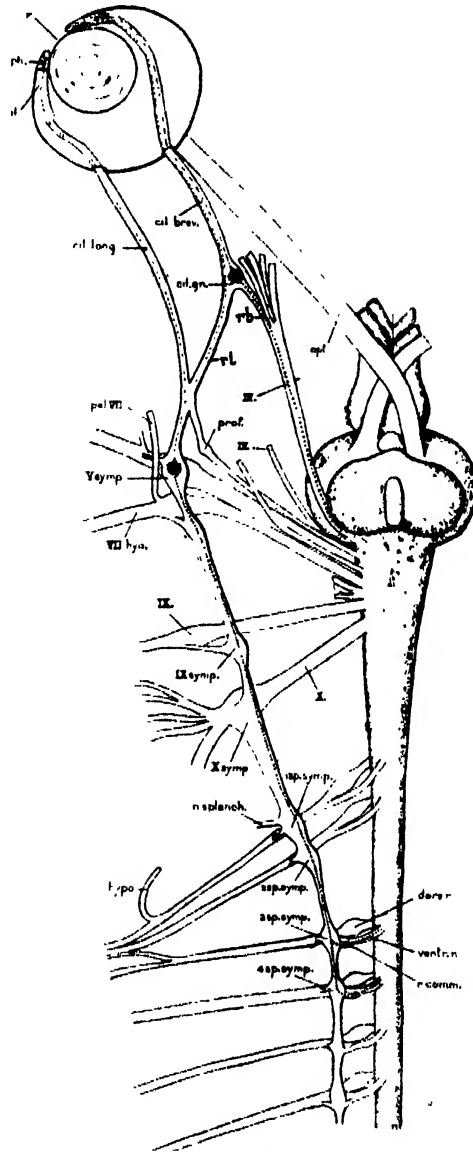
Stimulation was by means of an induction coil giving an alternating current; the stimuli used were always weaker than could be felt on the tip of the tongue. The electrodes were sometimes two fine platinum wires placed close together on the nerve, but more often one thick copper wire was tied into the muscles of the abdomen and the other electrode (very fine wire) applied to the nerve to be stimulated. *Uranoscopus* is a small animal and there is danger of mistaken results due to escape of the current through moist tissues. Control observations were always made in view of this possibility.

## 2. *Stimulation of the Sympathetic Nerves.*

In teleostean fish the sympathetic chain is continued into the head and bears a ganglion in connection with each of the dorsal cranial nerves. A full description of this chain and an analysis of its components is published in a separate paper (Young, 1931) to which reference can be made for further details. The general arrangement can be made out from the diagram on p. 469.

Stimulation of the sympathetic chain behind the head causes constriction of the pupil of the same side. This result was observed a great many times, using many different strengths of stimulus; never was there any sign of dilatation. After the stimulus has ceased the pupil returns approximately to its former size; prolonged stimulation, however, leaves the pupil narrower than its unstimulated fellow.

Stimulation of the roots of the 5th spinal nerves (the dorsal and ventral roots are too short to be stimulated separately) was never seen to cause changes in the pupil, but stimulation of the 3rd or 4th spinal roots always caused constriction. In the majority of cases stimulation of the sympathetic chain behind the 4th spinal nerve had no effect on the pupil, but in a few cases constriction was observed, probably due to escape of the current up the chain. The fibres which cause constriction, then, leave the C.N.S. and join the sympathetic chain in the white rami communicantes of the 3rd and 4th spinal nerves;



TEXT-FIG. 1.—Diagram of a ventral view of the sympathetic system of the front part of the body of *Uranoscopus*, showing the fibres concerned with the light reflex.

*cil. brev.*, short ciliary nerve; *cil. gn.*, ciliary ganglion; *cil. long.*, long ciliary nerve; *dil. m.*, dilatator pupillæ; *dors. r.*, dorsal root ganglion; *n. splanchn.*, splanchnic nerve; *opt.*, optic nerve; *p.*, pupil; *pal. VII.*, ramus palatinus facialis; *prof.*, profundus ganglion; *r. comm.*, ramus communicans; *r. b.*, radix brevis; *r. l.*, radix longa; *sph. m.*, sphincter pupillæ; *ventr. r.*, ventral root; *III.*, oculomotor nerve; *IV.*, trochlear nerve; *V.*, trigeminal nerve; *V. symp.*, trigeminal sympathetic ganglion; *VII. hyo.*, ramus hyomandibularis facialis; *IX.*, glossopharyngeus; *IX. symp.*, glossopharyngeal sympathetic ganglion; *X.*, vagus; *X. symp.*, vagal sympathetic ganglion; 1-4 *sp. symp.*, 1st-4th spinal sympathetic ganglia.

that they do not also leave in the 1st and 2nd spinal nerves was shown by other methods.

Microscopical examination has shown that medullated fibres do in fact join the sympathetic chain from the ventral roots of the 3rd and 4th spinal nerves and that these fibres run forwards in the chain (Young, 1931).

### 3. *Stimulation of the Oculomotor Nerves.*

In order to observe the effects of stimulation of the oculomotor nerve on the pupil it is advisable first to cut the extrinsic eye-muscles, otherwise the rapid withdrawal of the eye makes observation difficult. Stimulation of the oculomotor caused evident widening of the pupil in some individuals, in others it seemed to produce no effect. If in one of these latter cases the sympathetic chain was cut in front of the 3rd spinal nerve it was then found that stimulation of the oculomotor nerve caused dilatation of the pupil. Similarly in such cases stimulation of the oculomotor caused dilatation after cutting of the long ciliary nerve (in which some of the constrictor fibres run, see below) although not while all the nerves were intact.

### 4. *Stimulation of other Cranial Nerves.*

Stimulation of the common trunk of the trigeminus and facialis mid-way between the brain and the skull wall never caused any change in the pupil; but if the electrode was placed too near to the skull wall there was sometimes constriction due to escape of the current to the adjacent sympathetic ganglion; conversely stimulation near to the brain caused dilatation on account of escape to the oculomotor. The fact that no changes are seen in the iris on stimulation of the trigeminus and facialis is in agreement with the fact that few or no medullated (pre-ganglionic) fibres join the sympathetic from these nerves (Young, 1931).

### 5. *Stimulation of the Ciliary Nerves.*

Access to the radix longa and short ciliary nerves is best obtained by dissection from the ventral surface. Stimulation of either of these nerves caused constriction of the pupil.

The long ciliary nerve was reached from the dorsal surface by cutting the loose folds of skin behind the eye. The nerve runs alongside the superior rectus muscle and can best be stimulated after cutting the latter. In all cases stimulation of this nerve caused constriction of the pupil.

After the whole eye had been taken out, together with a length of the long ciliary nerve, stimulation of the latter still caused narrowing of the pupil.

This shows that the closure is due to the contraction of a sphincter muscle and excludes the possibility that it is due to dilatation of the blood vessels. This latter possibility was envisaged by Beer (1892) who even suspected that the narrowing of the pupil of the *isolated* eye might be a vascular phenomenon, the blood being drawn from the vascular glands at the back of the eye. This further possibility was also excluded in the present investigation by cutting away the whole of the back part of the eye, leaving only the iris and lens; stimulation of the stump of the long ciliary nerve then still caused contraction of the sphincter muscle.

After the long ciliary nerve had been cut, stimulation of the sympathetic chain still caused narrowing of the pupil, showing that some of the constrictor fibres run via the radix longa and short ciliary nerve. After the short ciliary nerve had been cut, stimulation of the sympathetic chain still gave narrowing of the pupil (via the long ciliary nerve), but stimulation of the oculomotor no longer caused dilatation. These experiments agree well with the results of microscopical examination, which showed that fibres run from the trigeminal sympathetic ganglion to both the long ciliary nerve and to the radix longa (Young, 1931).

#### 6. *Return of the Iris after Stimulation.*

If, after cutting either the sympathetic chain in front of the 3rd spinal nerve or the radix longa and the long ciliary nerve, the oculomotor nerve was stimulated, it was observed that the pupil dilated *and then returned to its previous state* as soon as the stimulus ceased. Now under these conditions all the nerve-fibres which produce constriction had been cut, so that there were no longer any complete motor neurons in connection with the sphincter iridis (with the exception of possible ganglion cells in the iris itself). The return after oculomotor dilatation was therefore a passive elastic effect on the part of the stretched sphincter, unaided by any tonic nervous impulses.

Conversely, after the oculomotor trunk or the short ciliary nerve had been cut, stimulation of the sympathetic chain produced narrowing of the pupil which was *still followed by dilatation* as soon as the stimulus ceased. The dilatator muscles had been passively stretched during the constriction and are capable of returning to their former state by reason of their own elasticity and independently of any motor, tonic innervation or of inhibition of the sphincter. This shows that it is not necessary to postulate an inhibiting effect of the dilatator nerves on the sphincter although it does not exclude the possibility that such exist (see Poos, 1927).

### 7. *Antagonistic Innervation of the Iris Musculature.*

Stimulation experiments have shown, then, that there is undoubtedly a double "antagonistic" innervation responsible for the control of the size of the pupil and that it works the opposite way round to that of Tetrapods. The sympathetic system provides the motor nerves to the sphincter muscle, the fibres leaving the central nervous system in the white rami communicantes of the 3rd and 4th spinal roots and thence passing forwards in the chain. So far there is nothing to indicate whether they have a synapse en route but there are constrictor fibres in both the long and short ciliary nerves. The dilator fibres run out in the oculomotor nerve and radix brevis to the ciliary ganglion and short ciliary nerve.

### BEHAVIOUR OF THE PUPIL AFTER DEATH.

Interesting confirmations and extensions of the above results were obtained from experiments involving the cutting of the nerves after the death of the animal; the preparation used was essentially the same as that already described for the stimulation experiments.

After an animal has been killed by cutting away the heart, etc., the pupils rapidly become very narrow and remain minimal for many minutes before gradually beginning to re-open. If one of the sympathetic cords was cut in front of the third spinal nerve during the period in which both the pupils were narrow, it was seen that the pupil immediately re-opened on the cut side; that is to say, the sphincter iridis had been released from the nervous influence which was producing the constriction.

This proved to be a useful method for localising the point of exit of the constrictor fibres from the C.N.S. During this period of *post-mortem* constriction the 5th and 6th spinal roots were cut on one side near to their point of exit from the vertebral column and no change was observed in the pupil. Next the 1st and 2nd spinal roots were also cut, still there was no change in the pupil, which remained as narrow as its fellow.

After a few minutes the 4th spinal root was cut and the pupil immediately became wider. Finally, after a further one or two minutes, the 3rd spinal root was also cut and the pupil became wider still. This experiment was repeated, with variations, five times, and it proves that the pupillo-constrictor fibres leave the C.N.S. in the rami communicantes of the 3rd and 4th spinal nerves and not in those of the 1st, 2nd or 5th nerves.

These experiments also show that the *post-mortem* constriction is due to

some centre in the central nervous system. This centre was localised more accurately. Transection of the medulla oblongata behind the auditory nerve or pithing of the hind part of the medulla and front part of the cord during *post-mortem* constriction had no effect on the pupils, which remained closed; therefore the constrictor centre does not lie in the brain. If, however, a wire was pushed further back down the spinal cord then both pupils immediately became wide. This shows that there is a true *centrum cilio-spinale inferior* (Budge's centre) lying at about the level of the 3rd and 4th spinal nerves and responsible for constriction of the pupil. Presumably the cell-bodies of the fibres which run out in the *rami communicantes* lie in this centre.

This *post-mortem* constriction of the pupil is comparable to the *post-mortem* colour changes of the skin which were observed in fish by v. Frisch (1911) and Wernøe (1926), and shown to be due to the motor impulses sent out to the sympathetic system by the dying cells of the central nervous system. It is curious that there seems to be no such agonal motor effect produced by the sympathetic cells themselves.

#### EXPERIMENTS WITH THE LIVING ANIMAL.

It was possible to confirm the results obtained from stimulation of the nerves by experiments on the living animal. *Uranoscopus* was found to lend itself well to such experiments except that the comparatively short time of survival in the aquarium made it impossible to follow the course of the degeneration of the nerves as far as could have been wished.

##### 1. *Methods.*

Most of the operations were slight and were performed without anaesthesia or special aseptic precautions. The animal was held in a damp cloth, if necessary with a circulation of sea water through a tube placed in the mouth. When the sensitive skin above the eye had to be cut the animals were anaesthetised by placing them in solutions of 4 per cent. magnesium chloride or 2 per cent. ethylurethane in sea water. Used alone these solutions act slowly and the best results were obtained by using them consecutively, in the above order.

##### 2. *Section of the Spinal Nerves and Sympathetic Chain.*

This operation was performed by means of a longitudinal dorsal incision to one side of the middle line, just behind the head. The operation was accompanied by very little bleeding and after sewing up the skin the animals showed



no ill effects. It was not possible to determine exactly what nerves had been cut until after the death of the animal, and in some cases the desired nerves were not cut; these failures serve as useful controls.

The following table gives the details of the operations performed:—

Nerves cut.	Result.
1. 3rd and 4th spinal roots ..	Light reflex abolished.
2.	
3. 3rd, 4th and 5th spinal roots.	
4.	
5. Sympathetic chain behind vagus .....	
6. Sympathetic chain between 3rd and 4th spinal nerves (3rd spinal root also damaged ?) .....	
7. 4th spinal root and sympathetic chain between 3rd and 4th spinal nerves .....	Light reflex impaired but not totally abolished.
8. 3rd spinal nerve peripheral to sympathetic chain and chain itself behind 4th spinal nerve.....	Light reflex unimpaired.
9. 3rd, 4th and 5th spinal nerves peripheral to sympathetic chain and chain itself behind 4th spinal nerve .....	
10. 3rd and 4th spinal nerves peripheral to sympathetic chain and chain itself behind 4th spinal nerve .....	
11. 5th and 6th spinal roots .....	

It will be seen that in six cases the 3rd and 4th spinal roots (or the sympathetic chain in front of these) were cut and that after such an operation the light reflex was no longer manifested on that side. The light reflex was left unaffected in three experiments, in which the 3rd and 4th spinal nerves were cut peripheral to the sympathetic chain and in one in which the 5th and 6th spinal roots were cut. In one experiment in which the 4th root was cut and the 3rd root left intact the light reflex was partly abolished. These operations thus confirm the conclusions previously arrived at, that the fibres responsible for the reflex run out in the 3rd and 4th spinal nerves.

After the death of the animals it was found that stimulation of the sympathetic chain at points behind the trigeminal ganglion caused no change in the pupil, whereas stimulation of this ganglion itself or of the sympathetic rami running forwards from it to the ciliary nerves caused narrowing of the pupil. It is evident, therefore, that the degeneration of the fibres only extended forwards as far as the trigeminal sympathetic ganglion; that is to say the pre-ganglionic fibres end there and the fibres in the long and short ciliary nerves are post-ganglionic.

The longest survivals after the operations were of 6 and 10 days and in these cases the nerves were removed, stained with osmic acid, and mounted for

microscopical examination. Degenerating fibres were seen as far forwards as the trigeminal sympathetic ganglion, in which they spread out among the cells. Unfortunately no preparations were made of the ciliary nerves.

No animals lived long enough for there to be any signs of paradoxical phenomena such as appear after similar operations on mammals.

### *3. Effect of the Sympathetic System on the Position of the Eye.*

After section of the spinal roots and sympathetic chain in the above experiments it was constantly noticed that the eyeball was abnormally protruded on the operated side. It was withdrawn when touched with a needle, but a stronger stimulus was required than was necessary to cause withdrawal of the normal eye. It was also frequently observed that stimulation of the sympathetic chain caused retraction of a normal eye, presumably by causing contraction of the extrinsic eye muscles. This is a remarkable fact and requires further investigation. For the present it can only be stated that fibres from the sympathetic do affect the position of the eye. It has been shown elsewhere (Young, 1931) that in *Uranoscopus* sympathetic fibres run to the wall of the orbit and to the oculomotor nerve and such fibres are also known to be present in mammals.

### *4. Section of the Oculomotor Nerve.*

This operation was performed by splitting open the dorsal wall of the skull so as to make an opening large enough for the introduction of a fine pair of scissors. The nerve was cut with one stroke and there was very little bleeding. The wound was closed by the application of either a small piece of softened paraffin wax, or of a wad of cotton wool, over which collodion solution was painted and allowed to harden. The skin is exceedingly thin over the top of the head and the wound cannot be closed by stitches.

Four successful operations were performed, all with similar results. If the operated animal was left for some time in the dark and then illuminated there was no apparent difference in the light reflex on the two sides. If, however, the tank was lit up after only a short stay in the darkness (so that the narrowing was less complete) then it was constantly observed that the pupil on the operated side closed more completely and re-opened more slowly than the normal pupil.

In two animals which survived for 7 and 18 days after the operation the nerves were cut out and stained with osmic acid. All of the fibres of the oculomotor were seen to be degenerating, but the radix brevis and ganglion ciliare consisted partly of degenerating and partly of sound medullated fibres

(Young, 1931). These latter originated in the trigeminal sympathetic ganglion and could be followed into the oculomotor nerve; the course which they take is certainly unusual, although fibres from the sympathetic to the oculomotor are well known in mammals.

The degenerating fibres in the radix brevis were the pre-ganglionic dilatator fibres which end in the ciliary ganglion, as is shown by the fact that no degenerating fibres were seen in the short ciliary nerve.

#### 5. *Section of the Trigeminus.*

The operation was performed in the same way as section of the oculomotorius, through a hole in the dorsal wall of the skull. Two experiments were made in which the trigeminus and profundus roots were cut, and one in which the whole profundo-trigeminus-abducens-facialis bundle was cut. The results were the same in all cases. At the moment of cutting the pupil of the same side became narrow but during the next two or three minutes it widened. Hereafter there was no detectable difference in the light reflex on the two sides under any circumstances (longest survival 4 days). The eye on the operated side was held constantly fully protruded and was not withdrawn when touched; presumably the afferent fibres for the reflex withdrawal pass in the trigeminus.

#### 6. *Section of the Ciliary Nerves.*

This was the most difficult of the operations performed since it involved opening the orbit. The animal was anaesthetised, a cut made in the loose skin of the postero-medial side of the eye, and the superior rectus muscle found. The long ciliary nerve runs alongside this muscle, and can be cut together with it; this was done in one experiment. It is possible, however, to find the nerve under the low-power microscope and to cut it without injuring the muscle. This was done successfully in two cases. The cut skin was then sewn up as far as possible and the wound closed with collodion. The eye was never completely normal after this operation and it could not be fully protruded, but there was no interference with the eye-ball or its blood supply.

The results of all three experiments were the same; for a while the light reflex was abolished and then afterwards slowly returned. In the experiment in which the superior rectus muscle had been cut, there was no sign of closure of the pupil of this eye for 6 days after the operation, but on the seventh and succeeding days there was undoubted closure. In the other two experiments, in which the superior rectus was left intact, the period of abolition of the reflex was much shorter, lasting only for 3 hours in the one and 4 hours in the other.

In all three experiments the closure in the operated eye was always less than in the normal eye. Evidently the temporary complete absence of the reflex was due only to operative disturbance and has no further significance.

These results agree with those obtained by stimulation of the nerves (p. 468) and with the anatomical investigations (Young, 1931), all going to show that the constrictor fibres run partly in the long and partly in the short ciliary nerves.

Cutting of the short ciliary nerve was attempted, but without success. The nerve lies deep in the orbit, surrounded by a jelly-like substance, and could not be separated from the ophthalmic artery and vein which run very close to it.

#### EXPERIMENTS WITH NICOTINE.

The local application of nicotine to sympathetic ganglia, or its injection, paralyzes the synapses between pre- and post-ganglionic fibres. Langley made much use of this drug in his analysis of the autonomic system of mammals but was unable to find any similar action in birds (1903). In the frog nicotine has the same action as in the mammal (see Schilf, 1926), and in teleosts Wernoe (1925 and 1926) found that application of 0.5 per cent. solutions to the sympathetic ganglia paralyzed the effect of stimulation of the spinal cord on the chromatophores.

Previous experiments having made it appear probable that there is, in *Uranoscopus*, a synapse in the trigeminal sympathetic ganglion, between the pre- and post-ganglionic neurons responsible for the narrowing of the pupil, it was desired to find out whether nicotine has a paralysing effect on this ganglion. Both local applications and injections were used.

##### *1. Local Application to the Ganglia.*

The animals were opened as described above and the heart and viscera removed. The sympathetic chains were dissected out on both sides and the skull opened ventrally. In order to reach the trigeminal sympathetic ganglion it was necessary carefully to split away the bones which surround it; this takes some time and it was not possible to expose both ganglia in order to use one as a control. The method adopted was therefore to paint sea water (or 50 per cent. sea water as the case might be) on to the ganglion and then, after an interval of some minutes (it having been shown that stimulation of the sympathetic chain still caused constriction), to apply the nicotine solution. This procedure was adopted in five experiments, and in all it was found that after the application of 1 per cent. solution of nicotine, stimulation of the

sympathetic chain no longer caused constriction of the pupil; that is to say, a synapse in the ganglion had been paralysed. In the remaining seven experiments the preliminary application of an isotonic solution to the ganglion as a control was omitted, it having been already shown that these solutions alone do not affect the reaction. In these experiments, however, nicotine was also applied to the anterior spinal, vagal and glosso-pharyngeal ganglia of the opposite side, and it was shown that this did not in any way affect the closure of the pupil as a result of stimulation of the 3rd and 4th spinal roots; that is to say, the constrictor fibres run through these ganglia without interruption. After the application of nicotine to the trigeminal sympathetic ganglion, the sympathetic roots of the ciliary nerves (i.e., the nerves in front of the trigeminal sympathetic ganglion) and the long ciliary nerve were stimulated and found still to cause constriction of the pupil.

The following is a specimen protocol:—

2.7.30.—Adult female *Uranoscopus*.

- 16.33. Both pupils wide; heart and viscera cut away and both sympathetic chains behind the head and the trigeminal sympathetic ganglion on the right side exposed.
- 16.50. R. and L. sympathetic chains stimulated behind the 2nd spinal nerves. Result: narrowing of the pupil.\*
- 16.52. Both pupils now equally half closed on account of the *post-mortem* constrictor effect produced by the C.N.S. (see p. 472).
- 16.53‡. 1 per cent. solution of nicotine in sea water painted on to the R. trigeminal sympathetic ganglion and on to the L. vagal and 1st and 2nd spinal sympathetic ganglia.
- 16.56. R. pupil nearly maximal, L. more than half shut. R. sympathetic chain stimulated behind 2nd spinal nerve, at level of vagus, glosso-pharyngeus and forwards to where it crosses the hyomandibular nerve. Result: in no case was there any narrowing of the pupil.
- 16.57. L. sympathetic chain stimulated behind 2nd spinal nerve. Result: narrowing of the pupil.
- 16.58. R. trigeminal sympathetic ganglion itself stimulated. Result: no change in the pupil.
- 17.01. R. orbit opened dorsally, m. rectus superior cut and long ciliary nerve stimulated. Result: narrowing of the pupil.
- 17.05. R. sympathetic chain stimulated behind 2nd spinal nerve, etc., as before. Result: no change in pupil. L. vagal sympathetic ganglion stimulated. Result: constriction of pupil.
- 17.08. R. sympathetic ganglion stimulated. Result: no change in pupil. R. radix sympathica stimulated (just in front of the trigeminal sympathetic ganglion). Result: narrowing of the pupil.

\* Stimuli were applied by "unipolar" electrodes, all stimuli being weaker than could be felt on the tip of the tongue (see p. 468).

Thus it is clearly shown that application of nicotine to the trigeminal sympathetic ganglion inhibits the constriction produced by stimulating the sympathetic more posteriorly, whereas when applied to the other sympathetic ganglia it has no such effect. It will be noticed that directly after the application of nicotine to the ganglion the pupil became wider than the control. This at first seems curious since in mammals the first effect of nicotine is to stimulate the cells of a ganglion to which it is applied, so that, by analogy, in this case, it would be expected that the pupil would become narrower. However, the ganglion is very thin and the cells lie only in layers two or three deep, so that the solution can penetrate very rapidly and possibly any initial stimulating effect is rapidly followed by paralysis. Further, it must be remembered that the pupil was already narrow as a result of the agonal effect of the C.N.S. (see p. 472). Paralysis of the synapse in the trigeminal sympathetic ganglion apparently releases the pupil from this constriction by cutting off the connection between pre- and post-ganglionic fibres, so that the net result, as soon as paralysis has set in, is dilatation of the pupil.

In several experiments (as also in the injection experiments described below) it was found that about 20 minutes after the application of the nicotine, stimulation of the sympathetic chain more posteriorly again began to cause constriction; it seems therefore that the effect of the nicotine wears off after a while.

## *2. Injection.*

No experiments were made in which the nicotine was introduced into the intact circulation. The method used was to cut the spinal cord behind the head, open out the abdomen, remove the viscera and to expose the sympathetic chains at the level of the 3rd and 4th spinal nerves on both sides. On one side the branchial arches were then cut through and the gills cut away; the heart and ventral aorta were laid bare and the afferent branchial vessels tied off on the cut side; if (in order to reach the sympathetic) the efferent branchial arteries were cut on the side with intact arches, they were also tied off. A syringe needle was then inserted through the bulbus arteriosus into the ventral aorta.

In this way a preparation was obtained in which only the arteries on one side remained in connection with the ventral aorta, so that an injection of nicotine could reach the trigeminal sympathetic ganglion of one side only.\*

Four experiments were made, the results being the same in all cases. Injection

\* There is a small transverse arterial connection in front of the brain but certainly the trigeminal sympathetic ganglion receives blood only from the arteries of the same side.

of 0.5 c.c. of 0.5 per cent. nicotine dissolved in sea water was enough to remove the constrictor effect of the sympathetic on the side with intact arteries, while leaving unimpaired the constrictor effect of the long ciliary nerve. After about 20 minutes, stimulation of the sympathetic again began to cause constriction (although, of course, the circulation having been stopped there was no question of the nicotine being washed away).

The following is a specimen protocol :—

- 17.7.30. Medium-sized female *Uranoscopus*.
- 19.00. Animal prepared as described above, the arches remaining intact on the left side.
- 19.26. Stimulation (unipolar) of the sympathetic chains behind the vagus on the right and left. Result : on both sides there is constriction of the pupil.
- 19.30. Injection of 0.5 c.c. of 0.5 per cent. nicotine in sea water.
- 19.31. Stimulation of the left sympathetic chain behind the vagus. Result. no change in the pupil. Repeat several times.
- 19.32. Stimulation of the right sympathetic chain behind the vagus. Result : constriction of the pupil.
- 19.35. Left orbit opened from the dorsal side. Long ciliary nerve stimulated. Result : constriction of the pupil.
- 19.36. Left sympathetic chain stimulated as before. Result : no constriction of the pupil even with a strong stimulus.
- 19.38. Stimulation of right sympathetic chain as before. Result : constriction of the pupil.
- 19.43. Repeat as at 19.36 with the same results.
- 19.46. Left sympathetic chain stimulated behind the vagus. Result : a very little constriction of the pupil.
- 19.49. Left sympathetic chain stimulated behind vagus. Result : slight constriction of the pupil.
- 19.55. Left sympathetic chain stimulated behind the vagus. Result : marked constriction of the pupil.

### 3. *Effect of Injection on the Ciliary Ganglion.*

An attempt was made to discover the effect of the injection of nicotine on the ciliary ganglion. The preparation was made exactly as for the last experiment but the skull was opened dorsally, allowing of stimulation of the oculomotor nerve inside the skull. After injection of nicotine, stimulation of the oculomotor no longer gave dilatation of the pupil on *either* side. This may have been due to communication of the arteries of the two sides in front of the brain. No method was devised for preventing this, so the experiments remain uncontrolled.

#### 4. *Application to the Ciliary Ganglion.*

The animal was prepared as described above, the orbit opened ventrally and the oculomotor nerve stimulated on both sides to show dilatation of the pupil. A small wad of cotton wool soaked in 1 per cent. nicotine dissolved in sea water was then applied to the ciliary ganglion of one side. After this application stimulation of the oculomotor within the skull no longer caused dilatation on the treated side. Stimulation of the short ciliary nerve, which had also been moistened with nicotine, still gave constriction of the pupil, showing that the nerve fibres had not been affected.

#### 5. *Conclusions from Experiments with Nicotine.*

It has been shown that the fibres producing contraction of the m. sphincter iridis leave the spinal cord in the 3rd and 4th spinal roots and run via the rami communicantes and forwards in the sympathetic chain, without a synapse, so far as the trigeminal sympathetic ganglion. Here they end and pass on the stimulus to the post-ganglionic neurons, whose fibres run via the long and short ciliary nerves to the iris.

The dilatator fibres leave the brain in the oculomotor nerves and run pre-ganglionic in the radix brevis to the ciliary ganglion. Here they have a synapse and fibres of the post-ganglionic neurons, run in the short ciliary nerves to the iris.

#### EFFECT OF DRUGS ON THE IRIS MUSCULATURE.

It is obviously desirable to test the effect of some of the usual drugs on this curious iris musculature, in which the sphincter receives its motor innervation from the sympathetic system and the dilatator from the oculomotor.

In mammals (see Poos, 1927) the application of sympathetico-mimetic drugs (adrenalin, etc.) causes contraction of the dilatator muscles and relaxation of the sphincter whereas parasympathetico-mimetics (pilocarpine, physostigmin) cause contraction of the sphincter.

The only previous investigations of the pharmacological reactions of the iris of fish appear to be those of Magnus (1899), who noticed that cocaine and pilocarpine increased the total time of narrowing of the pupil of the isolated eye in response to the incidence of light. He also observed that in frogs atropine and cocaine dilate the pupil, but that neither pilocarpine nor physostigmine constrict it. Beer (1894) observed that after injection of atropine the pupil became wider in certain Teleosts.



In the present study the cornea was removed and the solutions dropped gently on to the eyes from a pipette. The eyes were either left in the head or removed to capsules. The solution to be tested was dropped on to one eye and an isotonic solution on to the other as a control. The following table gives the results of the experiments.

Drug.	Results.
Pilocarpine (B.D.H.) (neutralised with HCl in sea water) 1 per cent. or 2 per cent. 0.5 per cent. to 0.05 per cent. ....	Immediate narrowing of the pupil until almost closed. Closure of pupil after 1 to 5 minutes. Very rapid closure of pupil.
Arecoline HBr (B.D.H.) 1 per cent. in sea water	Partial closure of pupil. Subsequent stimulation of the sympathetic or addition of pilocarpine or arecoline caused further narrowing.
Eserine sulphate (B.D.H.) 0.5 per cent. and 1 per cent. in sea water.	Partial closure of pupil. Subsequent addition of pilocarpine caused further narrowing.
Acetyl choline (Hoechst) 0.1 per cent. in sea water (solution 1 year old).	No apparent change in pupil. Subsequent stimulation of the sympathetic or addition of eserine or pilocarpine will not cause any narrowing.*
Atropine sulphate (Erba) 0.5 per cent. and 1 per cent. in sea water.	No apparent change in size of pupil in five experiments. Slight narrowing in four experiments. In all, paling of the chromatophores. Subsequent addition of pilocarpine caused narrowing of the pupil.
Adrenaline HCl† (Clin.) 0.05 per cent. and 0.025 per cent. in 50 per cent. sea water.	No apparent change in size of pupil. Paling of chromatophores. Subsequent addition of pilocarpine caused no narrowing (the chloretone contained in the preparation having paralysed the muscle).
Adrenaline HCl (Parke Davis) 0.05 per cent. in 50 per cent. sea water.	

\* If both pupils were first narrowed with pilocarpine or arecoline, and atropine was then dropped on to one of them it was observed that this pupil proceeded to re-open somewhat and to assume an elongated shape. Apparently the atropine inhibits the contraction produced by the other drugs; no explanation of the curious shape can be offered.

† Adrenaline (Clin.) was also injected (0.5 c.c. or 1 c.c.) into the muscles of the tail. The animals rapidly became pale all over and remained so for 24 hours. The pupils became slightly narrowed during 1 to 2 hours.

Thus the sphincter iridis of *Uranoscopus* contracts very rapidly when treated with pilocarpine or arecoline and more slowly with eserine and acetyl choline. These contractions, and also the contraction produced by electrical stimulation of the sympathetic chain, are inhibited by atropine. Adrenaline possibly has a slight constrictor effect. Although this muscle undoubtedly receives its motor innervation from the sympathetic system, yet it has all the pharmacological characteristics of the mammalian sphincter iridis, which receives its motor nerves from the oculomotor and is usually classed as a para-sympathetic muscle. In fact, the pharmacological reactions of the iris muscles are not necessarily associated with any particular innervation.

Grynfeltt (1910) has shown that the sphincter iridis of Teleosts, like that of mammals, consists of smooth muscle derived from the para iridiaca retinae. It is clear, then, that morphologically and pharmacologically the muscles are similar in the two groups and it remains to explain the curious difference of innervation, and to see whether this can be correlated with any difference in function.

#### DISCUSSION.

It is well known that in Teleosts the resting eye is accommodated for near vision, and that in active accommodation (for far vision) the whole lens is pulled nearer to the retina by means of a special retractor lentis muscle. Apparently the changes in size of the pupil during accommodation have not been investigated in fish. Beer (1894) noticed that in *Uranoscopus*, contraction of the sphincter iridis actually pushed the lens nearer to the retina (this was also noticed in the present work), and thus would help in far accommodation. By analogy with the mammals, in order to increase depth of focus, it would be expected that the pupil would be narrow for near (resting) vision and that it would become wider during distant accommodation. This seems on the face of it an unlikely sequence of events, and in fact there is some evidence that the resting pupil is wide and that during distant accommodation it becomes narrower. If a *Uranoscopus* is lying with its head close to the side of a lighted tank I have sometimes, but not always, observed that the pupils become narrower on my approach. Presumably previous to my appearance the eyes had been accommodated for near vision and this narrowing of the pupils accompanied a distant accommodation. It seems likely that there is some connection between the fact that the accommodation and its accompanying pupillary changes are the opposite in mammals and fish and that the mechanism for the control of the pupil is also reversed, but it is difficult to understand exactly what the connection can be. Unfortunately it is not known which nerves are responsible for the innervation of the m. retractor lentis of fish; in mammals the muscles of accommodation are probably innervated only from the oculomotor nerve and not also from the sympathetic system.

Even if it were possible to find a teleological explanation for the difference in innervation it yet remains extremely difficult to understand how two such opposite mechanisms can have been evolved. The similarities between the two are as baffling as the differences. It is in itself very curious that a centre for the control of the pupil should lie back in the spinal cord as does Budge's centre,

especially when it is remembered that the reflexes involved are almost all optic. Certainly in the mammals dilatation of the pupil occurs as a result of stimuli from many parts of the body, and this may be connected with the posterior position of the centre for dilatation (*cf.* Kapper's theory of neurobiotaxis). But in *Uranoscopus* constriction of the pupil seems to be produced mainly by impulses in the optic nerve, which must somehow be carried back as far as the 3rd and 4th sepal segments. In mammals the corresponding impulses pass to the oculomotor nucleus. It is hardly conceivable that two such similar mechanisms should have been independently evolved and yet how can a change-over have taken place?

In another paper (Young, 1931) it has been shown that the sympathetic ganglia in the head of Teleosts probably represent a secondary forward extension of the chain, and are not indicative of a primitive segmental arrangement of the cranial sympathetic system; this might perhaps explain the posterior position of the centrum cilio-spinale.

For the moment the problem remains unsolved. It is impossible to say which pupillary mechanism is the more primitive and whether one is derived out of the other or both from some simpler ancestral mechanism. It would be easier to understand the change-over if it was known for certain whether the oculomotor and sympathetic nerves to the iris of mammals contain only motor fibres to the sphincter and dilatator muscles respectively, or if they also contain inhibitory fibres to the other muscle as is argued by Poos (1927), though denied by most physiologists (Schilf, 1926). If there is indeed such a double innervation it is perhaps possible to imagine a change in function taking place, the inhibitory nerves becoming motor and vice versa.

A further study of the pupillary mechanism of other Teleosts is now being undertaken and may throw some light on the question. It may be that *Uranoscopus* is unique in the possession of this mechanism.

Further information might also be obtained by a study of the Cyclostomes, in which nothing is known about the mechanism of the eye, and of Selachians, which possess a ciliary ganglion but no sympathetic nerves to the iris, and whose pupils, nevertheless, are known to alter rapidly in size.

#### SUMMARY.

1. In the teleostean fish *Uranoscopus scaber* there is a well-developed light reflex. On illuminating one eye, the pupil narrows on this side only, there is no true consensual reflex. However, after cutting one optic nerve, bilateral illumination causes constriction in both pupils.

2. Electrical stimulation of the 3rd or 4th spinal roots, or of the sympathetic chain more anteriorly, or of the long or short ciliary nerves causes narrowing of the pupil.

3. Stimulation of the oculomotor nerves causes dilatation of the pupil.

4. After the animal has been killed by removal of the heart and viscera, the pupils become narrowed; they are released from this constriction by cutting the 3rd and 4th spinal roots, or the sympathetic chains anterior to these roots, or by pithing the cord. They are not released by destruction of the medulla oblongata. There is therefore a cilio-spinal centre, responsible for constriction of the pupil.

5. After unilateral section of the 3rd and 4th spinal roots of the living animal the light reflex is no longer manifested on this side, and the eye is more protruded than the control.

6. After section of one oculomotor nerve in the living animal the corresponding pupil narrows more readily and completely than the control.

7. By means of local applications and injections of nicotine it has been shown that there is a synapse of the constrictor fibres in the trigeminal sympathetic ganglion and of the dilatator fibres in the ciliary ganglion.

8. There is thus shown to be in this fish a well-developed nervous mechanism for the control of the pupil, resembling that of mammals in general plan, but with the functions of the "antagonistic" nerves reversed.

9. The pharmacological reactions of the m. sphincter iridis of *Uranoscopus* resemble those of the corresponding mammalian muscle.

#### BIBLIOGRAPHY.

- Beer (1894). 'Pflügers Archiv,' vol. 53, p. 523.  
 Brown-Séquard (1847). 'C. R. Acad. Sci.,' Paris, vol. 25, p. 482.  
 v. Frisch (1911). 'Pflügers Archiv,' vol. 133, p. 319.  
 Grynfeldt (1910). 'Bibliographie Anatomique,' vol. 20, p. 265.  
 Kuntz (1929). "The Autonomic Nervous System." London.  
 Langley (1903). 'J. Physiol.,' vol. 30, p. 221.  
 Langley and Orbeli (1910). 'J. Physiol.,' vol. 41, p. 450.  
 Magnus (1899). 'Z. Biol.,' vol. 33, p. 567.  
 Poos (1927). 'Arch. Exp. Path. and Pharm.,' vol. 126, p. 307.  
 Schilf (1926). "Das autonomen Nervensystem." Leipzig.  
 Steinach (1890). 'Pflügers Archiv,' vol. 47, p. 289.  
 Steinach (1892). *Ibid.*, vol. 52, p. 495.  
 Wernoe (1925). 'Pflügers Archiv,' vol. 210, p. 1.  
 Warnoe (1926). *Physiol. papers* ded. Aug. Krogh, p. 290.  
 Young, J. 'Quart. J. Micr. Sci.,' in the press.

612.826.3 + 612.014.44] : 612.015.3

*Metabolic Changes associated with Pigmentary Effector Activity and Pituitary Removal in Xenopus Lævis.*—I. *Respiratory Exchange.*

By ENID CHARLES, M.A., Ph.D., from the Department of Zoology, University of Cape Town.

(Communicated by H. H. Dale, Sec.R.S.—Received November 6, 1930.)

(a) *Introduction.*

There are various indications of connection between the pituitary gland and basal metabolism; between the pituitary gland and ovarian activity; and between ovarian activity and calcium metabolism. Progress in the study of the relationship of the pituitary to metabolism had been held back by the technical difficulties of mammalian hypophysectomy. By a method described by Hogben, the removal of the pituitary gland as a whole or of the anterior lobe alone can be performed without great difficulty and with great rapidity in adult Amphibia. The animals survive the operation indefinitely. The same author has shown that the posterior lobe of the pituitary gland plays a predominant rôle in controlling colour change, and latterly, in collaboration with Slome, has given strong indications that the anterior lobe of the gland also plays a part in determining the pigmentary effector activity of the South African clawed toad, *Xenopus laevis* (Daudin). These circumstances have suggested the advisability of parallel investigation of the effects of the total or partial removal of the pituitary gland and the influence of normal agencies affecting colour change (and thereby pituitary activity) upon the metabolism of this species. The present communication is concerned with two issues:—

1. Total respiratory exchange in air.
2. Dermal oxygen consumption in water.

The conditions affecting colour response, and the time relations of colour response, etc., in *Xenopus laevis*, have been recorded thoroughly (Slome and Hogben). These authors have shown that photic stimuli are the predominant natural agencies, the condition for pallor being that the field of vision is occupied by a light-scattering surface, and the condition for darkening that the field of vision is occupied by a light-absorbing surface. Eyeless animals are intermediate in hue but the condition of eyeless animals or animals kept in the dark has been shown to be physiologically different from intermediat

animals in a stage of transition from the white background to the black background response and *vice versa*. This conclusion, based on an analysis of the time curve, receives some confirmation from the data hereafter recorded.

For the reasons stated the following stocks of animals were employed : (1) Normal pale animals kept in white containers for at least 2 weeks before experiment ; (2) Normal dark animals kept in black containers for at least 2 weeks before experiment ; (3) Eyeless animals operated on at least 2 months previously ; (4) Hypophysectomised animals with both lobes removed operated on 3 to 12 months previously ; (5) Hypophysectomised animals with the anterior lobe only removed operated on 3 to 12 months previously ; (6) and (7) The same as (4) and (5), but eyeless. Parallel series of males and females for (1), (2) and (3) were employed to control sexual variation. For (4) to (7) females only, averaging about 70 gm. body weight were used. The reason for this is that females are considerably larger than males of the same age in *Xenopus laevis*, and it was desirable to obtain the maximum quantity of blood from operated animals. The method of operation has already been referred to. Animals in which the whole gland is removed remain indefinitely pale, and were kept continuously from the time of operation on a black background, to avoid the danger of using individuals in which any remains of the posterior lobe had been left behind. Animals in which the anterior lobe only had been removed were, for analogous reasons, kept on a white background, since it has recently been shown, by Hogben and Slome, that in *Xenopus* removal of the anterior lobe alone results in permanent expansion of the dermal melanophores. In the numerical data presented below, the standard deviation of the mean, *i.e.*,  $\sigma/\sqrt{n}$ , is given for each array of observations,  $\sigma$  being the standard deviation of the array.

The present investigation is particularly concerned with the relation of the pituitary body to metabolism. It has been shown by Hogben that removal of the pituitary in *Xenopus* is associated with involution of the ovary, and both these factors have been found to influence metabolism. Benedict and Homans found that removal of the hypophysis in dogs resulted in a drop in metabolism. Foster and Smith find the basal metabolism of totally hypophysectomised rats to be 35 per cent. below normal, and state that the rate can be restored to normal by injection of anterior lobe extract alone but not by posterior lobe extract alone. Winton and Hogben have studied the effects of hypophysectomy on the rate of carbon dioxide production in frogs and found a significant drop of from 21 to 29 per cent. of the normal value. With regard to the effect of involution of the ovaries, Loewy and Richter found a decrease in

absolute metabolism of 9 per cent. in castrated female dogs. Exactly how the removal of one or other of the ductless glands influences metabolism cannot be determined by respiration experiments alone. The possibility that muscle tone is an intermediary agent cannot be totally excluded by the employment of anaesthetised animals, since, as long as respiration persists the musculature involved must continue to function.

*Xenopus* is an animal of aquatic habit. It normally remains motionless at the bottom of a tank, coming up to breathe at intervals. If allowed free access to land, it does not normally avail itself of the opportunity of leaving the water, as do frogs kept in the same tank. Thus the total respiratory exchange of *Xenopus* is difficult to estimate in its normal surroundings. The facts that it will live for an indefinite period in moist air, if compelled to do so, and that it will remain submerged for long periods, makes it possible to circumvent this difficulty and, at the same time, to throw light on the relative contributions of the pulmonary and the dermal respirations to the total gaseous exchange. In this investigation the gaseous exchange of *Xenopus* in moist air, as measured by Haldane's method, is taken to represent the total respiratory exchange. The oxygen consumption of the submerged animal, determined by Winkler's method, gives the dermal respiratory rate. The difference between the two may be assumed to represent the pulmonary respiratory rate.

In setting forth the experimental data which follow, it is clear that the oxygen consumption per animal is not significant, since it is generally found that larger animals have on the whole a larger respiratory exchange than smaller animals. At the same time, if the oxygen consumption is expressed per gram or kilogram, it is found that it decreases as the size increases. A possible relationship has been suggested between heat production and the surface area of the body in animals. Empirically the surface law when applied to warm-blooded vertebrates yields fairly uniform metabolic rates. It is still uncertain whether it is appropriate to the respiratory rates of cold-blooded vertebrates. The data which follow are all presented in terms of unit surface, i.e., the total oxygen consumption divided by the two-thirds power of the body weight, and it will be discussed later whether this formula gives the most uniform results.

The previous condition of the animals was controlled by keeping all experimental animals under identical conditions in the laboratory and without food for at least a fortnight previous to experiment. Hill has shown that the heat production of frogs falls off during the first 2 weeks of fasting but afterwards remains approximately constant. The effect of external temperature on

respiration is in opposite senses in cold-blooded and warm-blooded vertebrates. In mammals the increased muscular activity at low temperatures results in an increased metabolism, while in cold-blooded vertebrates Krogh and Vernon have shown that there is a steady increase in metabolism as the external temperature rises. In the present series of experiments comparisons have been drawn only between data collected at practically identical temperatures, and the effect of temperature on the respiratory exchange of *Xenopus lævis* will be further discussed. Sexual differences do not affect the present investigation as males and females have been studied separately but data will be presented dealing with this point. Most observers have found a higher basal metabolism in males than in females. The question is complicated in *Xenopus* by the great discrepancy in weight between males and females, so that all possible relations between respiratory exchange and weight have to be considered before it can be definitely stated that the rate is greater in males than in females.

(b) *Respiratory Exchange in Air.*

(i) *Method.*—The respiratory exchange of *Xenopus lævis* in moist air was studied by means of Haldane's gas-analysis apparatus. This apparatus is well known and a detailed description is given in Haldane's "Methods of Air Analysis." Twenty-five determinations were made on outside air and the average oxygen and carbon dioxide percentages calculated. The results were, oxygen 21.015 per cent. and carbon dioxide 0.042 per cent. To determine the respiratory rate an animal was placed in a wire cage with a weight attached at the bottom for stability. The cage with animal was placed on a small glass Petri dish. This was placed on a  $\frac{1}{2}$ -inch layer of liquid paraffin in a large glass dish and covered with a bell jar of about 2½ litres capacity, fitted with a cork through which glass tubing with three-way tap was inserted. The apparatus was set up in outside air so that the oxygen and carbon dioxide content of the air at the beginning of the experiment could be taken to be the value determined from the set of experiments previously mentioned. Each experiment lasted for 4 hours and the temperature and barometric pressure were recorded. In the Haldane apparatus as supplied by the makers, the levelling tube containing mercury is held in a clamp and is intended to be lifted up and down by hand. In this investigation the levelling tube was adjusted by means of a holder sliding on a stand permitting of both coarse and also very fine adjustments. With this arrangement the levelling tube has to be raised by hand in each determination when the mercury is being



driven over as far as the bell jar tap. All other movements are performed rapidly, coarse adjustments by sliding the holder up and down the stand and fine adjustments very accurately by turning the small screw. A rubber bulb was used for aerating the water jacket effectively.

At the close of the experiment the free limb of the tap connected with the bell jar was connected to the free limb of the tap of the gas burette in the Haldane apparatus. The mercury in the gas burette was then driven through the connection beyond the bell jar tap and the tap then turned so that the bell jar and gas burette were in communication. The mercury was then driven backwards and forwards six times, from the bottom of the gas burette to the bell jar tap so that a mixed sample of air could be obtained. Finally, about 20.1 c.c. of air from the bell jar were collected in the gas burette and the oxygen and carbon dioxide percentages estimated. The differences between these and the values obtained for outside air were taken to be the percentages of oxygen consumed and carbon dioxide produced by the animal. In calculating the amounts of oxygen and carbon dioxide involved, the volume of air space in each bell jar used was measured with the cage and petri dish inside it, and allowing for the height of the liquid paraffin at the bottom. In every case the volume of the animal was measured and subtracted from the available air space. The volume of air was then reduced to the corresponding volume of dry air at 0° C. and 76 cm. pressure by Haldane's table. The amounts of oxygen and carbon dioxide in milligrams per hour were, as already stated, divided by the two-thirds power of the body weight of the animal and the results tabulated. Where the respiratory quotient differs considerably from unity, Haldane has shown that a correction is necessary on account of the change in the nitrogen percentage. In the present series of experiments it was found that this correction never amounted to more than about 0.5 per cent. of the total amount and consequently it was neglected. During all these experiments the animals as far as could be observed remained practically motionless at the bottom of the cage, except in the case of the eyeless animals. Owing to the different order of magnitude of the oxygen and carbon dioxide percentage and the smallness of the latter, the respiratory quotients as obtained by this apparatus cannot be regarded as having any great degree of accuracy. Nevertheless the considerable differences obtained between the respiratory quotients of different classes of animals may quite possibly be significant.

(ii) *Results*.—Observations of the respiratory exchange in air of *Xenopus* are summarised in Tables I and II. The standard deviations of all means are shown, with the exception of the mean temperatures. The differences in the

temperatures at which the experiments were carried out were very small, all lying within a range of 1.5° C.

The fourth class of animals in Table I, namely dark room animals, has not been previously described. These animals were normal females kept in total darkness for at least 2 weeks prior to experiment. They were placed in black containers inside a black box placed in a windowless basement room. After placing the animals in position the room was not entered again until the experiments commenced. Each animal was taken from its container and immediately placed in the experimental chamber. For the remainder of the time the experiment lasted, the same conditions prevailed as in all other experiments.

Table I.—Respiratory Exchange in Air of *Xenopus Lævis* (Female).

Description of animals.	No. of animals.	Mean temperature (° C.).	Mean weight (grams).	Mean respiratory quotient.	Mean O <sub>2</sub> consumption in mgm. per hour per W <sup>3/4</sup> .	C. of V. O <sub>2</sub> consumption.
						Per cent.
1. Hypophysectomised both lobes, eyed .....	12	17.2	56.3 ± 4.4	1.12 ± 0.13	0.117 ± 0.019	37
2. Hypophysectomised anterior lobe only, eyed .....	10	17.3	83.8 ± 10.4	1.06 ± 0.10	0.214 ± 0.022	33
3. Hypophysectomised both lobes, eyeless .....	10	17.5	70.2 ± 8.2	0.95 ± 0.10	0.280 ± 0.038	43
4. Dark room .....	12	17.5	44.0 ± 3.3	0.98 ± 0.09	0.310 ± 0.038	41
5. Normal pale .....	12	17.3	67.3 ± 4.2	0.81 ± 0.05	0.331 ± 0.021	22
6. Normal dark .....	12	17.2	57.0 ± 4.5	0.83 ± 0.03	0.397 ± 0.028	25
7. Eyeless .....	9	17.3	53.3 ± 4.6	0.79 ± 0.03	0.556 ± 0.059	32

Table II.—Difference in Respiratory Exchange of Male and Female *Xenopus* (White Background).

Sex.	No. of animals.	Mean temperature (° C.).	Mean weight (grams).	Mean respiratory quotient.	Mean O <sub>2</sub> consumption in mgm. per hour per W <sup>3/4</sup> .
Male .....	6	17.5	31.8	0.81	0.422
Female .....	12	17.3	67.3	0.81	0.331

The data presented in Tables I and II can be discussed under several headings.

(a) *Effect of Removal of one or both Lobes of the Pituitary.*—Animals having the anterior lobe only removed had a higher respiratory rate than those with both lobes removed and the difference may be significant. The difference between the mean rates of oxygen consumption is  $0.097 \pm 0.029$  mgm.

(b) *Difference between Hypophysectomised and Normal Animals.*—Comparing hypophysectomised animals with both lobes removed with normal pale animals, the difference in mean rates of oxygen consumption is  $0.214 \pm 0.028$  mgm., and comparing animals with anterior lobe only removed with normal dark animals, the difference is  $0.183 \pm 0.036$  mgm. Both these differences are clearly significant. It may therefore be stated that the effect of removal of one or both lobes of the pituitary is to reduce the respiratory exchange of *Xenopus* in air by about 50 per cent.

(c) *Respiratory Exchange of Animals kept in Darkness previous to Experiment.*—It will be seen that animals kept in darkness have a lower respiratory rate than normal animals kept in the light. They have a significantly higher rate of oxygen consumption than hypophysectomised eyed animals, the difference being  $0.094 \pm 0.044$  mgm. The difference being their oxygen consumption and that of normal pale animals is not significant. Comparing them with normal dark animals the difference in oxygen consumption is  $0.087 \pm 0.047$  mgm., which may possibly be significant.

(d) *Pale and Dark Animals.*—Animals kept on a black background have a higher respiratory rate than animals kept on a white background, the difference in rates of oxygen consumption being  $0.066 \pm 0.035$  mgm. This difference may be significant being nearly twice its standard deviation.

(e) *Eyed and Eyeless Animals.*—It will be seen that both in normal and hypophysectomised animals, eyeless animals have a higher respiratory rate than eyed animals, the difference in the former case being  $0.159 \pm 0.065$  mgm. It seemed probable that part of this difference might be due to increased movement as shown by the higher coefficients of variation of the eyeless animals. This will be considered subsequently.

(f) *Sexual Differences.*—The data presented indicate that males have a higher respiratory rate than females. Very few observations were made, but this point will be discussed at greater length in another section.

(g) *Coefficients of Variation.*—The coefficients of variation of a series of observations is defined as the percentage deviation of the mean, i.e.,  $100\sigma/M$ . The coefficients of variation of normal animals are 22 per cent. and 25 per cent. These compare favourably with those given by other workers on metabolism. Hill gives a coefficient of variation for heat production in frogs of

29 per cent. Winton and Hogben found a coefficient of variation (C of V) for carbon dioxide production in air of normal frogs of from 27 per cent. to 29 per cent. The higher coefficient of variation of hypophysectomised animals may indicate a greater amount of movement. If this were the case it would imply that the effect of hypophysectomy in lowering basal metabolism is even greater than is shown.

(h) *Respiratory Quotient*.—The method employed for determining respiratory exchange in air, as already stated, cannot be expected to give respiratory quotients of a high order of accuracy, owing to the difference in absolute amounts of oxygen and carbon dioxide measured, and the fact that small errors in the gas measurements produce a much larger relative error in the respiratory quotient. Nevertheless it will be seen that the average respiratory quotients of normal animals are fairly uniform, lying between 0.78 and 0.83, with standard deviations of about 5 per cent. The respiratory quotients of the hypophysectomised animals lie between 0.95 and 1.12, with standard deviations of about 10 per cent. The mean respiratory quotient of 22 hypophysectomised animals was found to be  $1.09 \pm 0.09$  and the mean respiratory quotient of 24 normal animals was  $0.82 \pm 0.03$ . The difference between these two is  $0.27 \pm 0.095$ . It may therefore be concluded that hypophysectomised toads have a significantly higher respiratory quotient than normal toads. It is believed that a respiratory quotient higher than 0.9 indicates that conversion and storage of fat from carbohydrate is taking place (Boothby and Sandiford). The ovaries are known to be intimately connected with fat metabolism and hypophysectomy in *Xenopus* has been shown by Hogben to be followed by marked involution of the ovary and enlargement of the fatty bodies. The high respiratory quotient of hypophysectomised animals thus falls into line with the known relationship between the pituitary body and the ovaries.

(c) *Dermal Respiration in Water*.

(i) *Methods*.—The animals employed in the following experiments had been kept in the laboratory under conditions identical with those already described for the experiments on respiration in air. The hypophysectomised animals had been operated on over a year previous to experiment. All animals had been starved for at least a fortnight. In each experiment the animal was first weighed and then placed in a small wire cage with a long wooden handle. The cage with animal inside was placed in a cylindrical glass vessel containing 1 litre of distilled water previously shaken up with air. The water was at a

temperature of 25° C. *Xenopus laevis* has its natural habitat in brackish water, hence it was thought that immersion in pure distilled water might be detrimental to the animal and 1 c.c. of sea water was added to each litre of distilled water. Liquid paraffin was poured on top of the water to form a layer about  $\frac{1}{2}$  inch thick and the vessel placed in a thermostat which maintained a constant temperature of 25° C.  $\pm$  0.5° C. After the animal had remained in the vessel for 1 hour, a sample of the water was syphoned into a bottle under paraffin and the oxygen content determined, a sample of the water with which the vessel was filled having been previously estimated. The form of syphon used has been described by Hogben and Zoond and was designed with a side piece of glass and rubber tubing. By this means water can be first run through the syphon tube and out of the side piece. Thus air bubbles are eliminated and contact between the water which passes over into the bottle and the atmosphere is reduced to a minimum. Blank determinations in which an experiment was set up and estimations made as usual but with no animal in the vessel gave a loss of oxygen of from 1 per cent. to 2 per cent. Two 100-c.c. samples were taken from the water before the animal was placed in it, and two at the end of the experiment and the oxygen content determined by the Winkler method. The difference between the means of the initial and final titrations multiplied by 10 gave the total amount of oxygen consumed during the period of the experiment. The Winkler method of estimating dissolved oxygen is too well known to necessitate detailed description. It was found to be unnecessary to use the Rideal Stewart modification for dealing with dissolved nitrates when distilled water was employed. Successive titrations of similar samples with N/100  $\text{Na}_2\text{SO}_3$  never differed by more than 0.1 c.c. The initial oxygen content of the water was from 8.0 mg. to 8.4 mg. per litre.

As a check on the method two experiments were performed in which the carbon dioxide production was estimated as well as the oxygen consumption. Controls were set up at the same time and 100 c.c. samples taken at the end of the experiment from both the control vessel and the one containing the animal. 10 c.c. N/100  $\text{Ba}(\text{OH})_2$  was added to each sample and the whole titrated with N/100 HCl. The two simultaneous determinations of oxygen consumed and carbon dioxide produced gave a mean value for the respiratory quotient of unity. These two experiments were performed as a check and are not put forward as giving an accurate value for the respiratory quotient.

(ii) *Results*.—In Table III the data from the experiments on dermal respiration are summarised. The number of animals used in each series, the mean oxygen consumption and the coefficient of variation for each series are given.

The data given indicate certain differences in dermal respiration between different types of animals.

(a) *Background*.—Animals kept on a white background have, in the case of both males and females, a higher oxygen consumption than those kept on a black background, but the difference cannot be shown to be statistically significant.

(b) *Eyed and Eyeless*.—Eyeless animals have a higher oxygen consumption than eyed animals. In the case of males the difference is  $0.017 \pm 0.01$  mg., and in females it is  $0.028 \pm 0.012$  mg. The difference between the means is thus about twice the standard deviation and the odds in favour of its being significant are about 21 to 1.

Table III.—Dermal Oxygen Consumption in Water, *Xenopus Lævis*.

Duration of Experiment = 1 hour. Temperature = 25° C.

Description of animals.	Number of determinations.	Mean weight in grams.	Mean $O_2$ consumption in mgm. per hour per $W^{2/3}$ .	Coefficient of variation.
				per cent.
Dark ♂♂ .....	24	$29.9 \pm 1.6$	$0.150 \pm 0.007$	23.0
Pale ♂♂ .....	24	$34.6 \pm 1.5$	$0.157 \pm 0.008$	24.9
Eyeless ♂♂ .....	15	$31.4 \pm 2.5$	$0.171 \pm 0.009$	20.5
Dark ♀♀ .....	14	$72.6 \pm 3.8$	$0.114 \pm 0.006$	19.8
Pale ♀♀ .....	22	$65.1 \pm 2.7$	$0.119 \pm 0.007$	27.7
Eyeless ♀♀ .....	12	$57.0 \pm 5.7$	$0.145 \pm 0.001$	26.3
Hypophysectomised both lobes ♀♀ .....	9	$57.3 \pm 6.6$	$0.094 \pm 0.009$	27.6
Hypophysectomised anterior lobe only ♀♀ .....	3	82.9	0.078	—
Hypophysectomised anterior lobe only, eyeless ♀ .....	1	55.8	0.181	—

(c) *Hypophysectomised and Normal*.—Hypophysectomised animals, those with both lobes removed and those with the anterior lobe only removed, have a lower oxygen consumption than normal animals. An exception occurred in the case of one eyeless animal with the anterior lobe removed which gave a high oxygen consumption. The number of hypophysectomised animals available at the time these experiments were carried out was not large enough to permit of adequate statistical treatment, but Table III shows that the difference in oxygen consumption is considerable and most probably significant. Comparing the 12 hypophysectomised females with the normal pale females, the mean oxygen consumption is  $0.090 \pm 0.008$  mgm. and  $0.119 \pm 0.007$

mgm. respectively. The difference is  $0.029 \pm 0.011$  mg., so that the hypophysectomised animals have a mean dermal oxygen consumption 24 per cent. lower than that of normal pale animals.

(d) *Sex*.—Males have a higher oxygen consumption than females in all cases. The differences between the means are: pale animals,  $0.036$  mg.  $\pm 0.009$  mg.; dark animals,  $0.038 \pm 0.011$  mg.; eyeless animals,  $0.026 \pm 0.014$  mg. The differences here appear to be significant.

(d) *The Relation of Temperature to Respiratory Exchange.*

It is universally found that a rise in external temperature is accompanied by a rise in the respiratory exchange of cold-blooded vertebrates. Krogh and others have found that the relation between respiratory exchange and external temperature can be described by van't Hoff's law governing the effect of temperature on chemical equilibrium. In the cases hitherto investigated the rise in temperature has been found to be very rapid so that between  $15^{\circ}$  C. and  $25^{\circ}$  C. the rate of respiratory exchange is at least doubled. In order to be able to compare the results of experiments performed at different temperatures, a number of determinations of oxygen consumption in air by the Haldane method were performed on *Xenopus* males kept under uniform conditions. The external temperatures varied from  $13^{\circ}$  C. to  $25.5^{\circ}$  C.

Table IV.—Oxygen Consumption of *Xenopus* (Male) at Different Temperatures.

Temperature. ° C.)	Number of determinations.	O <sub>2</sub> consumption in milligrams per hour per W <sup>2/3</sup> .
13.0	5	0.312
17.5	6	0.422
20.3	6	0.468
24.2	6	0.557
25.5	3	0.594

The results of respiration experiments at different temperatures are summarised in Table IV and are also shown graphically (fig. 1). Owing to the small number of determinations, the mean values for oxygen consumption given in the table have a very large probable error, consequently the curve drawn can only be regarded as a rough approximation to the probable relation existing between temperature and respiratory exchange in *Xenopus*. It would appear that the data obtained lie on a curve similar in type to those

described by other workers on Amphibia. The curve for *Xenopus*, however, rises less steeply, indicating that a rise in temperature of 10° C. is not accom-

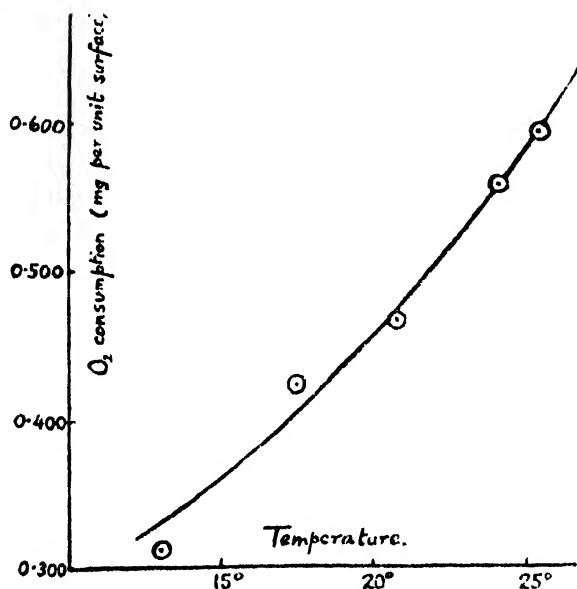


FIG. 1.

panied by as great a rise in respiratory exchange as has been described by Krogh in *Rana*. Such a difference is not without bionomic significance when it is borne in mind that the upper thermal death point of *Xenopus* is about 10° C. higher than that of *Rana*; and some observations made in this laboratory indicate that *Xenopus* die of asphyxia before reaching the temperature at which heat rigor supervenes. This suggests that the upper lethal temperature in *Xenopus* is due to the fact that its oxygen consumption has increased beyond the point where the oxygen carrying capacity of the blood, depressed by the rise in temperature, is no longer capable of meeting the requirements of the tissues.

(e) *Relation of Oxygen Consumption to Mass of Animal.*

Apart from temperature there is another point which calls for control in experiments of the type described in this section.

It has already been stated that while the surface law has been found to yield constant metabolic rates when applied to warm-blooded vertebrates, sufficient data are not available to warrant the conclusion that it has any significance when applied to cold-blooded vertebrates. While the point cannot be adequately discussed in the present communication, it seems desirable to make the following



comments. In fig. 2 points have been plotted of the average dermal oxygen consumption, divided by the two-thirds power of the body weight of normal animals having weights between 15 and 25 grams, 25 and 35 grams, and so on. Both males and females are shown, males being indicated by  $\odot$ , and females by  $\nabla$ . The graph summarises the results of 84 experiments. It will be seen that the oxygen consumption per unit surface falls as the weight of the animal increases. The surface law, when applied to *Xenopus*, does not yield a constant which is independent of the total mass of the individual.

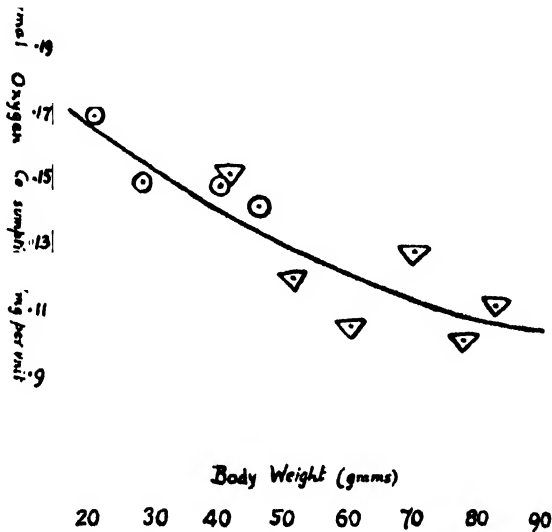


FIG. 2.

It can, however, be seen from an inspection of the tables given of the results of respiration experiments, that the differences observed between different classes of animals, with the possible exception of the sex difference, would still hold good whatever relation was found to exist between oxygen consumption and weight of animal. A tentative search was made for some relation which would yield a more constant rate of oxygen consumption. It was found that the difference between the respiratory rates of small and large animals was least when the oxygen consumption was divided by the cube root of the weight. When this was done the difference between the respiratory rates of males and females was reduced but still remained significant. Although in the figure given males and females appear to lie on the same curve it may therefore be said that there is a significant difference between the respiratory rates of males and females irrespective of the difference in size between them.

In fig. 3 curves are plotted for the same series of data as in fig. 2, but showing different relationships between oxygen consumption and weight. Arbitrary

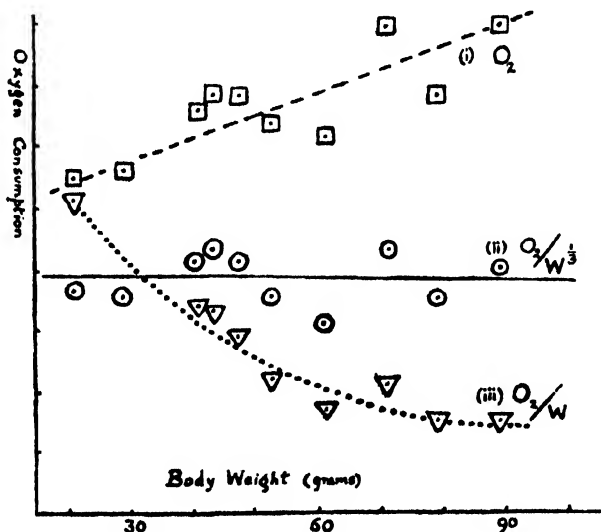


FIG. 3.

and different scales have been taken for oxygen consumption, in order to show the three curves at once, the figure being intended only to illustrate the form of the relationship. It will be seen that curve (i) of oxygen consumption rises steadily as mass of animal increases; that curve (iii) of oxygen consumption per unit mass falls more rapidly with increasing size than the curve shown previously in fig. 2 of oxygen consumption per unit surface; and that curve (ii) of oxygen consumption divided by the cube root of the weight approximates to a constant. The present investigator is unable to offer any theoretical explanation of this fact. In fig. 3 for the sake of clearness smooth curves have again been drawn through both male and female results, but nevertheless the slightly lower respiratory rate of females can be detected in all cases.

(f) *Ratio of Pulmonary to Cutaneous Respiration.*

With reference to the relation between pulmonary and cutaneous respiration, data have been previously placed on record by Krogh. From the figures given it is seen that in *Rana esculenta* the amount of oxygen taken in by the lungs is about equal to that taken in by the skin, whereas in *Rana fusca* it is about twice as great. To arrive at the relation in *Xenopus*, the oxygen consumption of males kept on a white background can be considered. The results

of determinations of dermal oxygen consumption in water at a temperature of 25° C. gave a mean value of 0.157 mg. The mean value for total oxygen consumption in air was obtained by interpolation from the graph given in the previous section of the oxygen consumption at different temperatures of similar animals. It was found to be 0.580 mg. By subtraction the pulmonary oxygen consumption was found to be 0.423 mg. The ratio of pulmonary to cutaneous oxygen consumption obtained from these figures is 2.7 to 1. It may therefore be concluded that in *Xenopus* the pulmonary oxygen consumption is from two and a half to three times greater than the cutaneous oxygen consumption.

(g) *Experiments on Anæsthetised Animals.*

Both in the determinations of total respiratory exchange and of oxygen consumption in water the eyeless animals were found to have a higher metabolic rate than normal animals, and the higher coefficient of variation suggested that this was due to greater activity. This would explain the anomalous circumstance that animals kept in a dark room till they give the same type of chromatic response as eyeless animals have a *lower* metabolic rate than the normal. To confirm this supposition some observations were made on animals anæsthetised with urethane. The oxygen consumption of a series of animals in one group of experiments was as follows :—

(Milligrams per W<sup>2/3</sup>.)

Eyeless.	Dark.	Pale.	Dark room.
0.152	0.275	0.321	0.124
0.256	0.247	0.228	0.134
0.233	0.193	0.198	0.154
0.203	—	—	—
Mean 0.211	0.238	0.249	0.137

The carbon dioxide production for five toads of each of the above series was :—

Eyeless.	Dark.	Pale.	Dark room.
0.233	0.267	0.231	0.183
0.235	0.389	0.260	0.202
0.217	0.233	0.223	0.158
0.311	0.277	0.231	0.216
0.279	0.229	0.266	0.193
Mean 0.255	0.279	0.242	0.190

It seems legitimate to infer that the high value of the eyeless animals in the foregoing experiments was due to greater muscular activity, and that the low value of animals kept for some time in a dark room is a genuine indication of a lower metabolic level. Since the hypophysectomised animals had an oxygen consumption lower than normal anaesthetised animals kept in the same conditions, and since the coefficient of variation of the hypophysectomised animals indicates that their activity was greater—rather than less—than normal, it is clear that the removal of the pituitary gland is accompanied by a lowering of the basal metabolism in *Xenopus laevis*.

(h) *Discussion.*

The main outcome of the present research is to show that the removal of the pituitary gland in Amphibia involves a reduction in the oxygen consumption of the organism and a higher respiratory quotient. Three points especially suggest themselves for further consideration: (a) by what structural constituents of the gland are these several effects determined; (b) through what agencies is their influence exerted; (c) can they be correlated with pigmentary effector activity.

(a) *Localisation of the Active Principles.*—Clawed toads in which the anterior lobe has been removed have a lower oxygen consumption than normal individuals. The oxygen consumption of individuals deprived of both lobes is lower than either, but is not significantly lower than that of individuals deprived of the anterior lobe alone. It seems clear that the anterior lobe is the main, and probably exclusive, agent in determining the metabolic phenomena studied in this communication. It should be added, however, that unpublished observations made in this laboratory suggest that in *Xenopus* as in *Urodeles* the anterior lobe includes both the pars anterior and the pars tuberalis of the mammalian gland.

(b) *Mode of Action.*—The activity of the anterior lobe is known to be associated with that of the thyroids and that of the ovaries. The clearest evidence of the influence of the anterior lobe on thyroid activity is supplied by P. E. Smith's work on Amphibian metamorphosis. Extracts containing the heat stable anterior lobe principle with which Hogben (1923) and later Spaul (1925) induced metamorphosis of axolotls, when injected into *Xenopus laevis* have been found in this laboratory to produce moulting of the skin such as follows the effects of thyroid feeding in *Urodele* larvæ. This may be taken as a strong presumptive indication of what Crew has called the thyreotropic influence of the anterior lobe. It is well established that thyroxin facilitates

tissue oxidation and thyroid removal is generally found to result in diminished oxygen consumption. The facts elicited do not prove that the secretory activity of the pituitary exerts a direct influence upon tissue oxidation. It seems more likely that the phenomena which have been established are attributable to the influence which the anterior lobe exerts upon the thyroid gland and the ovaries. To arrive at a final decision on this question it would be necessary to obtain data from castrated and thyroidectomised animals which had been deprived of the anterior lobe.

(c) *Metabolic Accompaniments of Pigmentary Effector Activity.*—The data presented in this communication suggest that metabolic differences are associated with the exercise of the chromatic function in so far as animals kept for long in the dark room have a lower oxygen consumption than normal animals. This confirms conclusions which Slome and Hogben have based on an analysis of the time relations of chromatic response in *Xenopus*. They are led to the view that the intermediate condition of the melanophores, in animals kept in darkness, is associated with a physiological state different from that which accompanies the intermediate condition of the melanophores during transition from the white to the black background response. Perhaps it is legitimate at this stage to proffer a purely speculative suggestion which might prove fruitful in other fields of enquiry. The analysis of the chromatic function in Amphibia has conclusively proved that the activity of the pituitary gland is reflexly stimulated by light. It is not impossible that this archaic relationship has been retained in animals which have lost the power of colour response, and that the pituitary gland may prove to provide a key to metabolic differences which do seem to be associated with the exposure of organisms to conditions of continuous illumination or darkness.

This research was undertaken at the suggestion of Professor Lancelot Hogben to whose encouragement and advice acknowledgment is made.

(i) *Summary.*

The conclusions of the foregoing investigation may now be summarised.

1. The rate of respiratory exchange of *Xenopus* in air increases as the external temperature is raised. The rate of increase is not as rapid as that found by previous workers in other species of Amphibia.

2. The rate of oxygen consumption per unit surface does not yield a constant independent of the mass of the animal. It decreases as the body weight increases.

3. The determination of sexual differences in the respiratory rate of *Xenopus* is complicated by the great difference in mean body weight in the two sexes. The data presented indicate that females have a lower respiratory rate than males which is independent of the size factor.

4. No significant differences were observed in the dermal respiratory rates of animals showing the black and white background response. Eyeless animals if not anæsthetised have higher rates of respiratory exchange than eyed animals, but this appears to be due to greater activity. Animals kept in total darkness prior to experiment have a lower respiratory rate than animals which have been kept in the light.

5. Removal of one or both lobes of the pituitary lowers considerably the rate of respiratory exchange. The effects of total and partial hypophysectomy do not appear to be significantly different, though animals in which the anterior lobe only has been removed have a higher respiratory rate than those in which both lobes have been removed.

6. The effect of hypophysectomy on the pulmonary rate of respiratory exchange is greater than its effect on the dermal respiratory rate.

7. Hypophysectomised animals have a significantly higher respiratory quotient than normal animals.

8. In *Xenopus* the rate at which oxygen is absorbed by means of the lungs is from two and a half to three times greater than the rate at which it is absorbed through the skin.

#### LITERATURE.

- Boothby and Sandiford (1924). 'Phys. Rev.,' "Basal Metabolism."  
 Benedict and Homans (1912). 'J. Med. Research,' vol. 25, pp. 409-502.  
 Foster and Smith (1926). 'J. Amer. Med. Assoc.,' vol. 87, p. 2151.  
 Haldane. "Methods of Air Analysis."  
 Hill, A. V. (1911). 'J. Physiol.,' vol. 43, pp. 379-394.  
 Enid Hogben and A. Zoond (1930). 'Trans. Roy. Soc.,' S. Africa, vol. 27.  
 Hogben (1923). 'Quart. J. Exp. Physiol.,' vol. 13, p. 2.  
 Hogben (1930). 'Proc. Roy. Soc.,' S. Africa.  
 Krogh. "Respiratory Exchange of Animals and Man."  
 Slome and Hogben (1928). "The Chromatic function in *Xenopus levis*," 'S. African Journal of Science.'  
 Winton and Hogben (1923). 'Quart. J. Exp. Physiol.,' vol. 12, p. 304.

612.826.3 + 612.014.44] : 612.015.3

*Metabolic Changes associated with Pigmentary Effector Activity and Pituitary Removal in Xenopus Lævis.—II. Calcium and Magnesium Content of the Serum.*

By ENID CHARLES, M.A., Ph.D., from the Department of Zoology, University of Cape Town.

(Communicated by H. H. Dale, Sec.R.S.—Received November 6, 1930.)

(a) *Introduction.*

The relationship between the parathyroids and calcium metabolism has been well established. Removal of the parathyroids in mammals results in tetany accompanied by a fall in the blood calcium content (Cruickshank, Salvesen, etc.). Tetanic symptoms and their fatal consequences can be prevented in dogs by an addition of calcium lactate to the diet (Luckhardt and Goldberg) and Collip has found that parathyroid extract raises the calcium content of the blood in both normal and parathyroidectomised dogs. Later work has raised doubts as to the specificity of the calcium effect of parathyroid extract. Davies, Dickens and Dodds obtained the same effects from injections of insulin and also pituitary extracts.

Contradictory results have been reported in connection with the thyroid, namely, a rise in calcium after thyroidectomy (Maxim and Vasilin), and a rise after thyroid feeding (Cahane). Mirvish and Bosman were able to produce a fall in blood calcium in rabbits both by injection of extract of suprarenal cortex and by injection of ovarian extract. Their work is in accord with that of Taylor and Caven who found a rise in serum calcium after double adrenalectomy, and that of Werner who found a rise after castration in guinea pigs and sheep.

The relationship of the pituitary gland to calcium metabolism has not been extensively investigated. Davies, Dickens and Dodds in their paper already quoted obtained a rise in serum calcium by means of injections of pituitrin. Nakazawa found that commercial extracts of each lobe had no effect on the calcium content of urine and fæces. It must, however, be borne in mind that many commercial extracts are known to lack some of the active principles of the pituitary gland.

Very little work has been done hitherto on magnesium metabolism. Luckhardt found that magnesium chloride administered *per os* prevented parathyroid

tetany, and in this connection it is suggestive that herbivorous mammals have a higher magnesium content than carnivorous mammals and that sheep are known to be more tolerant than many other mammals of parathyroidectomy (Sutherland Simpson).

In the present section calcium and magnesium metabolism will be considered in connection with pigmentary effector activity and the removal of one or both lobes of the pituitary gland.

(b) *Calcium Content of Serum.*

(i) *Method.*—As a sufficient amount of serum could not be obtained from one animal (*Xenopus*) for the determination of the calcium and magnesium content, a number of animals were bled at one time until enough blood was obtained to yield 9 c.c. of serum. The animals were bled by cutting through the pectoral girdle and exposing the heart. The pericardium was then removed and the heart and surrounding parts dried with filter paper to remove the pericardial fluid and the trematodes contained in it. The heart was then cut open and the blood collected in a beaker from which it was transferred to a test tube. A certain amount of hæmolysis took place in some cases, but, by centrifuging the serum for 20 minutes, a clear liquid was obtained which gave a clean precipitate when treated with ammonium oxalate.

The method adopted for the estimation of calcium in the serum was that of Kramer and Tisdall as modified by Clark and Collip and by Clark. 3 c.c. of *Xenopus* serum was taken for each estimation. As a check on the accuracy of the results the recovery of added calcium was tested with *Xenopus* and rabbit sera, using 3 c.c. samples of the former and 1 c.c. of the latter for each estimation. Enough serum of each type was taken to provide six samples and thoroughly mixed. To three of these, calcium chloride equivalent to 0.196 mg. Ca was added and the total calcium was then estimated in all six samples. Taking the average of each three estimations the results indicated that added calcium could be recovered from serum with a loss of not more than 3 per cent.

(ii) *Results.*—The results obtained from the estimations of the calcium content of *Xenopus* serum are shown in Table I.

The data so far obtained do not indicate any differences in the calcium content of the serum of pale and dark animals, or eyed and eyeless.

*Normal and Hypophysectomised Animals.*—Nine determinations on normal females gave a mean value of  $9.82 \pm 0.13$  mg. per 100 c.c. serum. The value for animals with both lobes removed is 7.22 mg., the difference being 2.2 mg.,



a drop of 26 per cent. The value for animals with anterior lobe only removed is 6.23 mg., the difference being 3.59 mg., a drop of 37 per cent. As the values for normal females all lie within a range of 1.1 mg. there appears to be a significant drop in the calcium content of the serum of hypophysectomised animals.

*Males and Females.*—Males were found to have a lower calcium content than females, the values being  $7.61 \pm 0.25$  mg. and  $9.82 \pm 0.13$  mg. respectively. The difference is  $2.21 \pm 0.28$  mg.

Table I.—Calcium Content of Serum. (Milligrams per 100 c.c.)

Eyeless. ♂ ♂	Pale. ♂ ♂	Dark. ♂ ♂	Eyeless. ♀ ♀	Pale	Dark ♀ ♀	Both lobes removed ♀ ♀	Anterior lobe removed ♀ ♀
8.6	8.36 6.89 7.25	6.75 7.73 7.72	9.85	10.23 10.00 9.19 9.16	10.10 9.61 10.14 10.10	7.22	6.23
Mean 8.6	7.5		9.85	9.65	9.99	7.22	6.23

(c) *Magnesium Content of Serum.*

(i) *Method.*—The method employed for the determination of the magnesium content of serum was that originally described by Bell and Doisy for estimation of phosphates, and later modified by Briggs and used by him for estimation of Mg. It depends on the fact that hydroquinone does not reduce  $\text{MoO}_3$  but does reduce phosphomolybdic acid. The magnesium is precipitated as magnesium ammonium phosphate. The precipitate is then converted into phosphomolybdate, treated with hydroquinone and sodium bisulphite and the resulting colour compared with a standard in a colorimeter. The chief difficulty with this method has been to obtain a stable blue colour. The present investigator found that the modification of the Bell and Doisy-Briggs method described by Benedict and Theis gives a blue colour which is stable for 24 hours after preparation. Denis has shown that the Bell-Doisy method is applicable to protein-containing serum.

The details of procedure are as follows. The magnesium content of the serum samples was estimated from the super-natant liquid after the precipitation of calcium. To obviate loss of supernatant liquid, the tubes containing the calcium oxalate precipitate were inverted over similar centrifuge tubes into

which the supernatant fluid had been poured. They were held in place by a strip of wood having holes in it screwed to the upper stage of a test tube rack so that the holes came immediately above the holes in the lower stage of the rack. They were left to drain in this position and in this way all but a trace of the supernatant fluid was recovered without disturbing the calcium precipitate. The magnesium was then precipitated by adding 1 c.c. ammonium phosphate, and 2 c.c. 10 per cent. ammonia, the sides of the tube very thoroughly scratched, and the precipitate left over night. The precipitate was then centrifuged, washed with 10 per cent. ammonia, centrifuged a second time, washed with ammoniacal alcohol, and centrifuged a third time. The times of centrifuging and the process of drying and draining were the same as previously described for calcium estimation. The present author found after trial that a second washing with ammonia as usually recommended did not appear to be necessary. The final precipitate was dissolved in 10 c.c. 0.1 N. HCl and the contents of the tube transferred to a 25 c.c. volumetric flask. 10 c.c. distilled  $H_2O$  were added, then 2 c.c. of the molybdate reagent and 2 c.c. of the hydroquinone-bisulphite reagent. The flask was filled with water up to the mark. At the same time a suitable amount of a standard  $KH_2PO_4$  solution was placed in a 25 c.c. flask and treated in the same way. All the flasks were heated for 10 minutes in a steam oven at a temperature of  $80^\circ$  to  $90^\circ$  and after cooling compared in a Duboscq-Leitz colorimeter. The standard solution used was such that 10 c.c. were equivalent to 0.07838 mg. magnesium. For *Xenopus* serum either 7 c.c. or 10 c.c. of the standard were taken. For the first determination in a series of similar samples, standards of different strengths were made up simultaneously and the most appropriate one used thereafter.

*Accuracy of Method.*—A series of six estimations carried out on a solution of magnesium chloride of known strength gave an average value within 1.4 per cent. of the calculated amount while the extreme errors were + 4 per cent. and - 6 per cent. Added magnesium was recovered from rabbit and crawfish sera with errors of 2 per cent. and 1.7 per cent.

(ii) *Results.*—The results obtained from estimations of the magnesium content of *Xenopus* serum are summarised in Table II.

The data referring to the magnesium content of the serum are closely parallel with those obtained for calcium.

*Normal and Hypophysectomised Animals.*—Seven determinations on normal females gave a mean value of  $2.35 \pm 0.10$  mg. per 100 c.c. serum. The value for animals with both lobes removed is 1.72 mg., the difference being 0.63 mg., a drop of 27 per cent. The value for animals with anterior lobe only

removed is 1.68 mg., the difference between 0.67 mg., a drop of 29 per cent.

*Males and Females.*—Males were found to have a lower magnesium content than females, the values being  $1.60 \pm 0.09$  mg. and  $2.35 \pm 0.10$  mg. respectively. The difference is  $0.75 \pm 0.13$  mg.

Table II.—Magnesium Content of Serum. (Milligrams per 100 c.c.)

Eyeless ♂ ♂	Pale ♂ ♂	Dark ♂ ♂	Eyeless ♀ ♀	Pale ♀ ♀	Dark ♀ ♀	Both lobes removed ♀ ♀	Anterior lobe removed ♀ ♀
---	1.24	1.72	2.63	2.78	2.20	1.72	1.68
---	1.54	1.68	---	2.07	2.03	---	---
---	---	1.80	---	2.20	2.51	---	---
Mean .....	1.39	1.73	2.63	2.35	2.25	1.72	1.68

(d) *Seasonal Changes in Serum Calcium and Magnesium.*

The foregoing experiments were made in the hot season, i.e., in February. Later determinations made in the months of July and September show that there is a seasonal variation both in the magnesium and calcium content of the serum of *Xenopus laevis*. The detailed statements of these observations will be set forth in a subsequent publication. Here it may be mentioned that the hypophysectomised animals were in all cases found to have a lower calcium content than normal animals. The magnesium content of the serum of hypophysectomised animals was not found to be lower than that of normal ones in July and September.

It is thus necessary to speak for the present with some caution regarding the effects of pituitary removal on the magnesium content of the serum. The blood of hypophysectomised animals in the winter months (July to September) was found to hæmolyse more readily than the blood of normal animals. This might give rise to fictitious values for the magnesium content of the serum.

(e) *Discussion.*

The outcome of the present research and the preceding communication of the same series is to show that the removal of the pituitary gland in *Amphibia* produces profound and lasting metabolic changes, which involve a reduction in the oxygen consumption of the organism and in the calcium and magnesium content of the serum. It would thus appear that the pituitary gland in the

normal animal exercises directly or indirectly a controlling influence on oxidative processes on the one hand and ionic equilibrium on the other. The calcium and magnesium content of the serum of individuals from which the anterior lobe only has been removed was found to be lower than that of individuals from which both lobes had been removed. The number of samples, however, does not permit of the conclusion that this difference is significant. It seems clear, however, that the "anterior lobe" is the main, and probably exclusive, agent in determining the metabolic phenomena studied both in this and the preceding communication; but there is some reason to suspect that in *Xenopus* as in *Urodeles* the anterior lobe includes both the pars anterior and the para tuberalis of the mammalian gland.

There are good reasons for believing that the activity of the anterior lobe is associated with that of the ovaries. In a preliminary note Hogben has called attention to the fact that removal of the pituitary in *Xenopus laevis* results in involution of the ovaries, while implantation of pituitary glands induces ovulation in females at periods several months before and after the breeding season. The data presented in this communication show that normal females have a higher calcium and magnesium serum content than normal males, and data derived from the study of sexual differences in other species reinforce the conclusion that the ovary exerts an influence on calcium and magnesium metabolism similar to that which the anterior lobe has been shown to exercise. It is therefore a suggestive fact that the calcium and magnesium content of the serum of hypophysectomised females is reduced to the same level as that of normal males. Against this, it must be mentioned that Mirvish and Bosman obtained a lowering of blood calcium by injecting ovarian extracts into rabbits. To arrive at a final decision on this question it would be necessary to obtain data from castrated and thyroidectomised animals which have been deprived of the anterior lobe.

The data presented in this communication do not afford evidence that pigmentary effector activity is associated with changes in the calcium and magnesium concentration of the blood.

(f) *Summary.*

1. Females were found to have a higher calcium and magnesium content of serum than males.
2. No certain correlation could be detected between calcium and magnesium content and pigmentary effector activity.

3. Removal of either the anterior lobe alone or of both lobes of the pituitary gland in *Xenopus lævis* is followed by a persistent fall in the calcium content of the serum.

This investigation was undertaken at the suggestion of Professor Lancelot Hogben to whose advice and encouragement acknowledgment is made.

#### LITERATURE.

- Bell and Doisy (1920). 'J. Biol. Chem.,' vol. **44**, p. 1.  
Benedict and Theis (1924). 'J. Biol. Chem.,' vol. **61**, p. 63.  
Briggs (1922). 'J. Biol. Chem.,' vol. **52**, p. 349.  
Briggs (1924). 'J. Biol. Chem.,' vol. **59**, p. 255.  
Cahane (1929). 'C. R. Soc. Biol.,' vol. **100**.  
Clark (1921). 'J. Biol. Chem.,' vol. **49**, p. 487.  
Clark and Collip (1925). 'J. Biol. Chem.,' vol. **63**, p. 461.  
Davies, Dickens and Dodds (1926). 'Biochem. J.,' vol. **20**.  
Denis (1922). 'J. Biol. Chem.,' vol. **54**, p. 693.  
Halverson and Bergheim (1918). 'J. Chem. Soc.,' Abstracts 114, p. 123.  
\*Hogben. "The Comparative Physiology of Internal Secretion."  
Kramer and Tisdall (1921). 'J. Biol. Chem.,' vol. **47**, p. 3; vol. **48**, p. 1.  
Mirvish and Bosman (1927). 'Quart. J. Exp. Physiol.,' vol. **18**.  
Mirvish and Bosman (1929). 'Brit. J. Exp. Biol.,' vol. **4**.  
Nakazawa (1928). 'Biochem. Z.,' vol. **198**.

\* For other references in the text.

*Studies on the Flexor Reflex.—I. Latent Period.*

By J. C. ECCLES, M.A., D.Phil., Research Fellow of Exeter College, and  
Sir CHARLES SHERRINGTON, O.M., F.R.S., Physiological Laboratory,  
Oxford.

(Received January 2, 1931.)

[PLATES 35, 36.]

## I. INTRODUCTION.

The latent period of the flexor reflex was first measured as the interval between the application of a stimulus and the beginning of the mechanical response of the muscle (Sherrington, 1906). Jolly (1911) introduced the electrical response of the muscle recorded by the string galvanometer as a basis of measurement of latent period, and he calculated that the time occupied in the passage through the spinal cord (central reflex-time) was about  $4\sigma$ . Forbes and Gregg (1915) used the electrical response of the motor nerve in their measurements of latent period and calculated that the central reflex-time was 3 to  $5\sigma$ , thus confirming Jolly. In both calculations, however, certain assumptions had to be made as to the velocity of propagation of impulses in the afferent nerve fibres.

Since 1915 there has been no systematic investigation of the latent period of the flexor reflex. In the following investigation an attempt has been made to throw fresh light on the nature and duration of its latent period.

## II. METHOD.

The general description of experimental technique given here applies to all the papers of the present series.

The animal (cat) having been anaesthetised, the spinal cord was transected at the 12th thoracic segment, and the 6th and generally the 7th post-thoracic dorsal roots were divided extradurally. Decerebration by the trephine method followed immediately and the anaesthetic was then discontinued.

Tibialis anticus muscle has been reflexly excited by stimuli applied usually to the popliteal nerve, but sometimes to the posterior tibial, hamstring, or internal saphenous nerves. All the nerves of the limb except the branches from peroneal to tibialis anticus have been cut peripherally, and all the afferent nerve fibres of peroneal have been cut centrally. In animals with a "post-fixed" sacral plexus this has been done by dividing

extradurally the 6th and 7th post-thoracic dorsal roots and by cutting in the sacral plexus the branch which 8th may send to peroneal. In animals with a normal or "pre-fixed" sacral plexus only the 6th post-thoracic dorsal root has been divided, and the branch which 7th sends to peroneal has been cut in the sacral plexus. The object has been to preserve a pathway to the spinal cord for impulses set up in the afferent fibres of popliteal nerve, despite complete deafferentation of tibialis anticus muscle.

By means of drills through both ends of the tibia the preparation has been rigidly fixed to the cast-iron top of the experimental table by uprights and clamps. The "frictionless" pattern (Eccles and Sherrington, 1930, *b*) of the isometric mirror myograph has been employed for registering the muscular contractions. A Cambridge string galvanometer has simultaneously registered the electrical responses on the same photographic plate. The bright myographic image records photographically on the less bright background from the galvanometer optical system. Time has been registered by a tuning fork having a frequency of 100 d.v. per second. Ag-Ag Cl leads have been used for leading off the electrical responses of the muscle. The sensitivity of the string (silvered quartz  $2.5 \mu$  diameter, 3900 ohms resistance) has been about 30 metres per ampere. Its frequency of vibration at that tension is about 350 d.v. per second. Plate 35, fig. 1, shows the effect of breaking a current of  $3 \times 10^{-6}$  amps. passing through the string.

All measurements have been made from photographic records taken by a falling plate camera at a plate-speed of about 450 mm. per second. The following method of measurement has been found to be both speedy and accurate. The plate is turned face downwards on accurately squared graph paper thereby avoiding errors due to parallax. The illumination is direct, not transmitted. Since the light traverses the plate twice, contrast is heightened and all lines sharpened, and with practice it is possible to judge distances to the nearest 0.05 mm. Since the plate has a speed of about 450 mm. per second, this means that time can be measured to the nearest 0.1  $\sigma$ .

Shielded electrodes have been applied to the intact motor nerve (peroneal) and usually fluid (blood) electrodes have been applied to the central end of the cut afferent nerve. By means of an electro-magnetically released Lucas pendulum with three break-keys, break induction shocks at known intervals have been supplied from three induction coils (coreless). These were placed at right angles to each other and at long distances apart so as to avoid effects of crossed induction. Calibrations of the Lucas pendulum performed at the beginning of the research and towards its end showed agreement within 0.1  $\sigma$  for intervals less than 40  $\sigma$ . The break-shocks applied to the intact motor nerve have in all cases been rather stronger than maximal for the motor nerve fibres (about 20 cm. coil distance with 0.4 amp. in the primary). In no case have the stimuli applied to either afferent or efferent nerves been sufficiently strong to be perceptible to the tongue. The strength has never been sufficient to set up more than one impulse in any nerve fibre. The rectal temperature of the preparation has been taken at intervals throughout all experiments and has almost always been between 35° C. and 38° C.

*Post-mortem* examination has been performed in all cases in order to ascertain if the operation of deafferentation had been complete, and in most cases the afferent and efferent nerves have been dissected out and the distances from the cathodes of the electrodes to the points of origin from the spinal cord have been measured on the unstretched nerves.

## III. EXPERIMENTAL INVESTIGATIONS.

1. *The Measurement of the Latent Period of the Flexor Reflex.*

(i) *Procedure.*—A single centripetal volley\* is set up by a break-shock which is applied to the afferent nerve through electrodes *c* (fig. 1). Several sigmata (usually about 20  $\sigma$ ) after the resulting reflex discharge has passed down the motor nerve to the muscle, the motor nerve is stimulated in continuity by a break-shock through electrodes *a*. Both break-shocks are produced by a Lucas pendulum at a known interval apart. The electrical response of the muscle is recorded by means of a string galvanometer and the interval between the beginning of the reflex action-current and the beginning of the motor action-current is measured to the nearest 0.1  $\sigma$ . This interval is identical with the interval (measured at the cathode of electrodes *a*) between the passage of the foremost impulses of the reflex discharge and the setting up of the motor impulses. The difference between this interval and the stimulus-interval (known from Lucas pendulum) is the time between the setting up of the centripetal volley at the cathode of electrodes *c* and the arrival of the foremost reflex impulses at the cathode of electrodes *a*, i.e., it is the shortest latent period of the reflex between these two points.

This method should be more reliable than methods where the instant of application of the stimulus to the afferent nerve is registered by photographing the break-key, because it avoids any errors arising from instrumental delay.

\* A centripetal volley is a collective term for the centripetal impulses (one only in each nerve fibre) set up in an afferent nerve by a single brief stimulus, e.g., an induction shock. A stronger stimulus sets up nerve impulses in fibres which could not be excited by a weaker stimulus, i.e., it sets up a centripetal volley having more nerve impulses. Such a centripetal volley is said to be larger than that set up by the weaker stimulus. Thus, when speaking of a large or small centripetal volley, it is the number of impulses which is used as the basis of comparison, not the size of each individual impulse, which, of course, is independent of the strength of the stimulus.

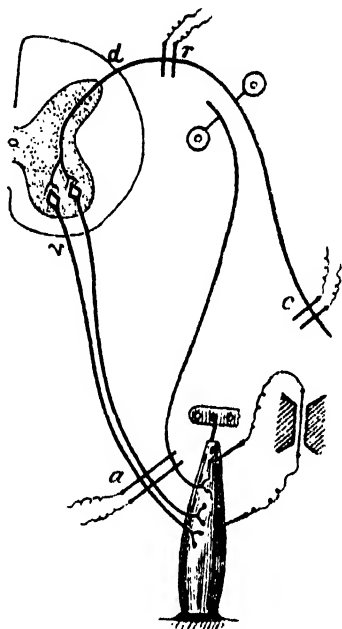


FIG. 1.—Schema of the reflex pathway and the recording systems.



Also any time taken by the break-shock to set up the centripetal volley at electrodes *c* cancels out with the time taken to set up the motor impulses by the break-shock at electrodes *a*.

(ii) *Experimental Results*.—In Plate 35, fig. 2, the stimulus-interval was  $31.8\sigma$ , and in the upper record the time between the beginning of the reflex action-current and the beginning of the motor action-current was  $21.4\sigma$ . The shortest latent period was therefore  $31.8\sigma - 21.4\sigma = 10.4\sigma$ . In Table II, column 4, are a series of similar measurements, which are in all cases the average of a number of values. If experimental conditions are kept constant, *e.g.*, the temperature of the animal and the strength of the stimulus applied to the afferent nerve (see p. 517), the value obtained for the latent period is found to be remarkably constant in any particular experiment. Variations rarely exceed  $\pm 0.2\sigma$  and so are almost within the limits of experimental error. In all cases electrodes *c* have been applied to the popliteal nerve in the middle of the thigh, electrodes *a* have been placed on the peroneal nerve at the knee joint, and the action-currents have been recorded in the tibialis anticus muscle.

## 2. *The Velocity of Propagation of Impulses in the Afferent and Efferent Nerve Fibres.*

In order to determine how much of the latent period is consumed in travel through the peripheral nervous paths and how much is central, *i.e.*, within the spinal cord, it is necessary to measure the velocity of propagation of the impulses in both the afferent and efferent nerve fibres and the lengths of the respective paths.

(i) *The Afferent Nerve Fibres*.—Only approximate figures have been available for the central reflex-time, because the velocity of propagation of those centripetal impulses which evoke the flexor reflex has been unknown. Measurements of the rate of propagation of impulses in the afferent nerve by means of the electrical response of the nerve are unreliable, since there is no guarantee that the values obtained belong to the centripetal impulses which evoke the flexor reflex. The only method available for measuring the velocity of propagation of these centripetal impulses in the afferent nerve is to determine the decrease in the latent period effected by shortening the length of the afferent path.

So as to make use of the longest possible path, electrodes *r* (fig. 1) are placed on that dorsal root (usually the 8th post-thoracic) through which the impulses set up by electrodes *c* enter the spinal cord (other alternative roots being previously cut). The latent period of the reflex path from electrodes *r* to

electrodes *a* is determined by the method just described, and then, in order to allow for the slight cooling of the spinal cord incident on the exposure, another series of determinations is made using electrodes *c*. The difference between the average *ca* latent period and the average *ra* latent period gives the time taken by the fastest centripetal impulses (which evoke the reflex) in travelling from *c* to *r*.\* The distance *cr* is measured on the unstretched nerve trunk dissected out at autopsy.

In the lower record of Plate 35, fig. 2, the shortest latent period was  $6.7 \sigma$ . Therefore the shortest time of propagation of the reflex impulses from *c* to *r* was  $10.4 \sigma - 6.7 \sigma = 3.7 \sigma$ . As the distance *cr* was 12.0 cm., the fastest velocity of propagation was 32.4 metres per second.† In this experiment the average of a series of determinations was 31.6 metres per second.

(ii) *The Efferent Nerve Fibres*.—The velocity of propagation along the motor nerve fibres has been ascertained by stimulating the 7th post-thoracic ventral root by a single break-shock at a certain interval ( $20 \sigma$  or more) before the application of a break-shock through electrodes *a*. After a series of such observations another series is taken with the pendulum keys set in the same position, but the break-shock from the earlier is now applied by electrodes *a*. The average interval between the two motor action-currents of the muscle is calculated for each series. The difference between the respective intervals is twice the time of travel from the cathode of the electrodes on the motor root to the cathode of electrodes *a*. This distance is measured in the same way as for the afferent pathway, and the velocity of propagation is calculated.

In Table I, columns 3 and 4, are all the measurements which we have made on the velocity of propagation in the afferent and efferent nerves respectively. The variations from experiment to experiment are not related to the temperature of the nerves and cannot be explained by experimental error, which is not greater than  $\pm 5$  per cent. for the afferent nerves and  $\pm 3$  per cent. for the efferent nerves. It can be concluded that at  $34^\circ \text{C}$ . the velocities of propagation in the fastest afferent fibres of the flexor reflex are and in the fastest efferent fibres are usually  $35 \pm 5$  metres per second, and  $80 \pm 15$  metres per second, respectively. The latter value accords well with the velocities determined by Erlanger and Gasser (1924) in the dog and cat for the  $\alpha$  fibres of the

\* This deduction is permissible only if the reflex response elicited by the stimulus at *r* is approximately the same as that elicited at *c* (see p. 517). In practice the error is minimised by making both reflexes fairly large; but the strength of the stimuli must not be sufficient to introduce an error due to the spread of current along the nerve fibres.

† In making this calculation no allowance is made for any delay in the spinal ganglion (Lenninger, 1913; Erlanger, Bishop and Gasser, 1926).

Table I.

Date of experiment.	Temperature at hip alongside nerves.	Fastest velocity of propagation of impulses in the afferent nerve in metres per second.	Velocity of propagation of impulses in the motor nerve in metres per second.
	° C.		
16.7.30	Not taken	20	Not measured
17.7.30	34	32	93
21.7.30	33.5	35	65
18.9.30	33	40	80
23.9.30	35	37	66
29.9.30	33.5	32	97
1.10.30	34.5	34	84
3.10.30	36	39	82

tibial and internal saphenous nerves. The former value of  $35 \pm 5$  metres per second for the fastest fibres of the afferent group often falls between the values given by Erlanger and Gasser (1930) for the slowest fibres of the A group (rarely less than 35 metres per second) and the fastest fibres for the B group (rarely more than 20 metres per second).

### 3. The Central Reflex-time.

By the central reflex-time is meant the time consumed in traversing the central reflex pathway, *i.e.*, through the spinal cord from the point of entrance of the afferent roots to the point of exit of the efferent roots. In all the experiments of Table II the total lengths of both the peripheral afferent path *ad* and the peripheral efferent path *va* were also measured. The respective conduction-times are shown in Table II, columns 5 and 6, and the total peri-

Table II.

1	2	3	4	5	6	7	8
Date of experiment.	Rectal temperature.	Temperature at hip alongside nerves.	Shortest latent period.	Time in afferent nerve.	Time in motor nerve.	Total conduction time in peripheral path.	Shortest central reflex-time.
	° C.	° C.	$\sigma$	$\sigma$	$\sigma$	$\sigma$	$\sigma$
16.7.30	35	Not taken	10.2	4.5	2.1	6.6	3.6
17.7.30	Not taken	34	10.4	4.4	2.1	6.5	3.0
21.7.30	—	33.5	9.05	3.55	2.65	6.2	2.85
18.9.30	34.5	33	10.55	3.8	2.4	6.2	4.35
23.9.30	36	35	9.65	4.1	2.8	6.9	2.75
29.9.30	35	33.5	10.2	4.6	2.0	6.6	3.6
1.10.30	36.5	34.5	10.6	4.15	2.2	6.35	4.25
3.10.30	37	36	10.3	3.65	2.4	6.05	4.25

pheral conduction-time in column 7. The central reflex-time in column 8 was calculated by subtracting the values of column 7 from those of column 4. Since the values in columns 4, 5, and 6 were calculated from a series of observations made in the course of only a few minutes, and were controlled for the effect of slight changes of temperature, the value obtained for the central reflex-time should be reliable to  $\pm 0.2 \sigma$ . The extreme values of this series are  $2.75 \sigma$  and  $4.35 \sigma$ ,\* and there is good agreement with the values calculated by Jolly (1911) and by Forbes and Gregg (1915).

#### 4. *The Effect of Alteration of Stimulus Strength on the Latent Period of the Flexor Reflex.*

If the strength of the stimulus applied to an afferent nerve is decreased, the resulting reflex response is found to show an increase of latent period as well as a diminution of tension (*cf.* Buchanan, 1907, 1908). This is shown in fig. 2, where the tensions of a series of reflex responses (ordinates) are plotted

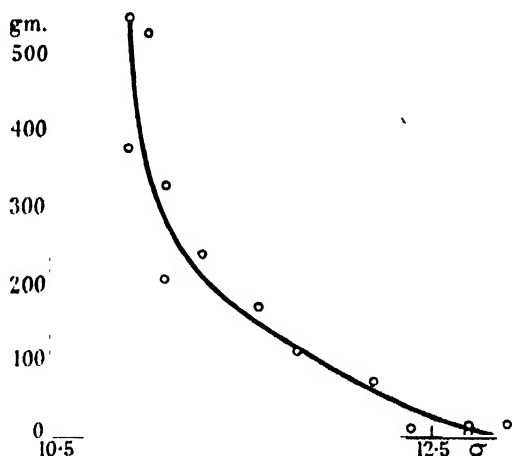


FIG. 2.—Tension of reflex responses (ordinates) plotted against their respective latent periods (abscissae).

against their respective latent periods (abscissae). Part of the series is shown in Plate 35, fig. 3. The observations were made at about half-minute intervals and in such an order that any progressive change in the preparation was

\* In experiment 23.7.30 the central reflex time was probably as long as  $7 \sigma$  even when the reflex responses were moderately strong. These observations were not included in Table II because the time in the afferent and efferent nerves was not accurately known. The diminution of the latent period by facilitation (*see* p. 523) was, however, almost  $7 \sigma$  (Table III), so the central reflex-time must normally have been still longer.

controlled. Many series of observations similar to that shown in fig. 2 have been obtained in all the other experiments (four in all) where a similar investigation has been attempted. In them the maximum alteration of latent period has usually been about  $2\sigma$ , but in one experiment it was little more than  $1\sigma$ .

The increase in the strength of the stimulus applied to the afferent nerve will have two results: impulses will be set up in additional nerve fibres, and, owing to the spread of current, impulses will be set up further from the stimulating electrodes in those nerve fibres originally excited by the weaker stimulus.

The alteration in the latent period is too great to be explained in this latter way. The influence of the spread is probably negligible, because the strongest stimuli which were employed (usually 16 cm. coil distance in a coreless coil) were relatively quite weak (not perceptible to the tongue). Moreover the change in the strength of the stimuli in fig. 2 was much greater for the six reflexes with the shortest latent period (16 cm. to 23 cm. coil distance) than it was for the remaining observations (23 cm. to 27.5 cm.).

Therefore most of the diminution of the latent period effected by the stimulus must be due to the impulses which are set up in the additional nerve fibres. Since the velocity of propagation in these afferent fibres is less than in the originally excited fibres (for the former have a higher threshold), the shortening of the latent period does not occur in the conduction-time of the afferent path. Also the shortening cannot occur to any appreciable extent in the conduction-time of the efferent path, because the velocity of propagation of nerve impulses in the efferent fibres shows but little variation from fibre to fibre (Erlanger and Gasser, 1924; see p. 523). *Therefore the shortening of latent period must be at the expense of the central reflex-time, and must be due to the additional centripetal impulses set up by the stronger stimulus.*

There are two possible explanations of how this can come about: either the motoneurones which are excited to discharge by a small centripetal volley respond with less delay when they are excited by a larger volley; or the additional motoneurones which are excited to discharge by the larger centripetal volley respond sooner than those which are excited by a smaller volley as well. Later (p. 529) it will be found that the observations of this and the succeeding sections are susceptible to a common explanation. This explanation accords with the former of the alternatives suggested above.

### 5. *The Temporal Dispersion of the Reflex Response evoked by a Single Centripetal Volley.*

(i) *Introduction.*—The contraction of a flexor muscle evoked reflexly by a single centripetal volley in an ipsilateral afferent nerve differs in both the electrical and mechanical records from the maximal motor twitch. The tension of the reflex response is usually less than that of the motor twitch, but it may be more. In this latter case the reflex discharge must excite some of the motor units more than once, *i.e.*, there is a repetitive discharge from some of the motoneurons (Sherrington, 1921, *a* ; 1921, *b*). This is also shown by the electrical record (Adrian and Bronk, 1929 ; Adrian and Forbes, 1922). When the tension of the reflex response is less than the maximal motor twitch, it can usually be distinguished from a submaximal motor twitch of similar tension by the following criteria :—

1. The action-current of the reflex tends to be more irregular than that of the motor twitch and is more spread out, *e.g.*, there is a longer time from the beginning of the action-current to the summit of the deflection.
2. The maximal amplitude of the deflection of the reflex action-current is generally less than that of the motor (of similar tension), often much less.
3. The reflex may show action-currents for some time after the main response (after-discharge).
4. The mechanical record of the reflex muscular contraction often has a longer "contraction-time" (*cf.* Cooper and Eccles, 1930), and may give evidence of after-discharge.

The first and second differences show that in the reflex response the contraction of the individual motor-units as a group has greater temporal dispersion than is the case in the motor response. This feature, as Forbes and Gregg have pointed out (1915), might be due either to the latent periods of the responses of different units varying greatly though being each single, or to some motor-units responding two or more times. The longer "contraction-time" of the reflex response is likewise significant of greater temporal dispersion.

(ii) *Experimental Procedure.*—The following experiment enables a decision to be reached between the above (Forbes and Gregg) alternative explanations of the temporal dispersion of the reflex discharge. Electrodes *a* (fig. 1) are placed on the intact motor nerve so that this can be stimulated in continuity. A single maximal break-shock applied through these electrodes sets up a single volley of nerve impulses which, on reaching the muscle, excites it to

**FIG. 3.**

spinal cord (assumed to be 1 cm.) ;  $va$  is the length of the motor path from the spinal cord to electrodes  $a$  ;  $am$  is roughly the length from  $a$  to the muscle  $m$ . As the abscissæ represent time in sigmata as shown in the scale, horizontal lines drawn through any point on the reflex arc show that point at different times.

A centripetal impulse set up at  $c$  will be represented by an oblique line  $cd_1$  drawn so that  $\tan cd_1d$  equals the velocity of propagation (assumed to be 35 metres per second). After the time-interval shown by  $d_1v_1$ , the shortest central time of the reflex, the foremost reflex discharge is shown by the oblique line drawn from  $v_1$ . Its slope is steeper than  $cd_1$  owing to its faster rate of propagation (assumed to be 80 metres per second). At a point  $a_1$ , just before  $v_1a_2m_2$  is due to reach electrodes  $a$ , the motor nerve is stimulated by a maximal break-shock through these electrodes. The antidromic volley so set up is represented by the line  $a_1v_4$  drawn so that  $\tan a_1v_4v$  equals the velocity of propagation, and the centrifugal volley to the muscle is shown by  $a_1m_1$ .

In that nerve fibre (in fig. 3 impulses in that fibre are represented by continuous lines) which conveys the foremost impulse of the reflex discharge (shown by  $v_1a_2m_2$ ), there will be a collision between this impulse and the antidromic impulse at the point  $x$ , and mutual extermination will occur, as shown by neither continuous line passing the point  $x$ . Similarly in each other nerve fibre along which an impulse of the reflex discharge travels, there will be a collision between that impulse and the antidromic impulse in that fibre resulting in mutual blocking. A reflex impulse which did not leave the spinal cord until  $4\sigma$  after  $v_1$  is shown by  $v_3y$ , and collision in that fibre occurs at  $y$  (the impulses in that fibre are shown by the line of crosses).

The line of circles  $a_1v_4$  represents an antidromic impulse in a fibre in which there is no reflex discharge or in which the reflex discharge leaves the spinal cord more than  $4.3\sigma$  after  $v_1$ . This antidromic impulse is free to pass into the spinal cord, and it has been shown that such an impulse blocks all reflex discharge from the motoneurone which it reaches for at least  $4\sigma$ , that this central blocking is just as effective for the purposes of the experiment as the block in the peripheral nerve paths by direct collision of impulses, and that no additional factors are thereby introduced (Eccles, 1931). The earliest reflex discharge from that motoneurone (which is possible under the circumstances) is represented by  $v_5m_4$ , which is more than  $8\sigma$  after the foremost reflex discharge  $v_1x$ .

Since in any nerve fibre the antidromic impulse is blocked by the first centrifugal (reflex) impulse, it has no effect on any of the subsequent impulses



in that fibre. For example  $v_2a_3m_3$  represents a second reflex discharge in the same nerve fibre along which  $v_1x$  had previously passed and blocked the antidromic impulse at  $x$ . Consequently  $v_2a_3m_3$  is unobstructed in its passage to the muscle.

Thus a single antidromic volley, when suitably timed, is able to block the whole of a reflex discharge even when this has a temporal dispersion as long as  $8\sigma$ , provided that there is no more than one reflex discharge from any one motoneurone. Therefore the experiment allows the following conclusions to be reached: -

(a) If the antidromic volley blocks all reflex discharge, there is no more than a single reflex discharge from each motoneurone.

(b) If the antidromic volley fails to block all reflex discharge, either:

- (i) there is more than one reflex discharge from some motoneurones; or
- (ii) there is no more than one reflex discharge from each motoneurone, but some motoneurones discharge impulses more than  $8\sigma$  before others, i.e., there is a temporal dispersion of more than  $8\sigma$ .

This second alternative seems improbable, but it is difficult to exclude it as a possible explanation of some of the unblocked reflex discharge.

(iv) *Results*.—Any reflex discharge which is not blocked by the antidromic volley will produce an additional response in the muscle after the maximal twitch and this should be apparent both from the mechanical and electrical records.

It is found that, with many reflexes, both the mechanical and electrical records fail to reveal any such additional response, and this may happen even when the reflex exhibits a considerable temporal dispersion. It must be concluded that, in these cases, more reflex discharges than one occur in very few if any motor-units, because the mechanical record of the maximal motor twitch is usually sufficiently constant to allow detection of an additional response in 1 per cent. of the motor-units, i.e., at most 5 units.

Thus a centripetal volley, initially synchronous, can give rise to a reflex discharge of an asynchronous volley of impulses, of which there is no more than one impulse discharged from any motoneurone. The resulting response of the muscle, because it is single in each individual unit, may be conveniently termed a *reflex twitch*. It can be distinguished from a submaximal motor twitch of similar tension only by its greater temporal dispersion.

Those reflex responses which are not completely blocked by a suitably timed

antidromic volley will be discussed in a later paper under the heading of *After-Discharge* (Eccles and Sherrington, 1931, *b*, p. 586).

A convenient measure of the temporal dispersion of the reflex response is the time by which the interval between the beginning of the reflex electrical response and the summit of its deflection, exceeds a similar interval for the motor response. Values between  $1\sigma$  and  $3\sigma$  are usual. This period does not represent the total temporal dispersion of the reflex discharge, but it is equal approximately to the difference between the shortest latent period and the "modal" latent period. The shape of the action-current of the reflex twitch frequently shows that there are also individual reflex paths with latent periods longer than the "modal" latent period. These are not taken into account in the above method of measuring temporal dispersion.

No appreciable temporal dispersion occurs during travel along the efferent pathway, for the time to the summit of the motor action-current is the same whether it be elicited by stimulating the ventral (motor) root or the motor nerve near the muscle. Therefore the temporal dispersion of the reflex response of a muscle indicates a similar temporal dispersion in the reflex discharges of the motoneurons. An explanation of how this comes about will be suggested later (see p. 529).

#### 6. *The Latent Period of the Response to the Second of two Centripetal Volleys.*

The observations of the next paper (Eccles and Sherrington, 1931, *a*) show that, when one centripetal volley follows another at a short interval, the motoneurons responding to the second volley can be divided into two groups, according as they have or have not also responded to the first volley. In the interpretation of the observations which follow, it is important to distinguish between these two groups of motoneurons (*cf.* p. 531). When there is a longer interval between the two centripetal volleys, the group responding to both volleys in succession increases (owing to the passing off of the refractory period following the first discharge), and the other group diminishes (owing to the decrease in the extent of the subliminal fringe following the first volley).

(i) *Both Centripetal Volleys in the same Afferent Nerve.*—In some experiments, when there was a short interval between the two centripetal volleys (called  $C_1$  and  $C_2$ ), the action-current of the response to the first volley,  $C_1$ , interfered with the action-current of the response to the second,  $C_2$ , in such a way as to make it difficult to locate the beginning of the second response, *e.g.*, the responses of Plate 35, fig. 4, with  $4.0\sigma$  and  $5.9\sigma$  intervals. But in other experiments the beginning of the second response was quite sharply marked

at a  $C_1C_2$  interval of  $5.9\sigma$ , *e.g.*, Plate 36, figs. 5 and 6. In the former the beginning of the second response occurred  $3.4\sigma$  after the beginning of the first, and in the latter this interval was only  $1.8\sigma$ , though in both  $C_2$  was set up  $5.9\sigma$  after  $C_1$ . Therefore in the former the latent period of the fastest part of the second response was  $2.5\sigma$  less than the fastest part of the first response, and in the latter it was  $4.1\sigma$  less. It will be seen later (see p. 526) that the shortening of the latent period of the response to  $C_2$  diminishes as the  $C_1C_2$  interval is increased. For the present, however, we are only concerned with the observations at that  $C_1C_2$  interval where there was the maximum shortening.

The results of experiment 23.9.30 will now be considered in detail. The latent period of the responses to both the first and second centripetal volleys could be determined from the same record as the shortening of the latent period, because the motor nerve was also stimulated (see p. 513) at a known interval after the setting up of the centripetal volleys (the large motor action-current is seen after the reflex action-currents in Plate 36, fig. 5). After a series of such observations the velocities of propagation of impulses in the afferent and efferent nerve fibres was immediately determined as on p. 515. Throughout the whole experiment the rectal temperature was repeatedly observed and was found to remain between  $36.0^\circ\text{C}$ . and  $36.2^\circ\text{C}$ .

In fig. 4 the average values of a series of observations are represented in a diagram similar to fig. 3,  $v_1a_1m_1$  and  $v_2a_2m_2$  are the foremost reflex discharges evoked by the first and second centripetal volleys,  $c_1d_1$  and  $c_2d_2$  respectively, when the  $C_1C_2$  interval was  $5.9\sigma$ . The slope of  $c_1d_1$  is equivalent to a velocity of 37 metres per second (average of 5 determinations), and  $c_2d_2$  is drawn at a similar slope, because it will not be affected by the refractory period following  $c_1d_1$ . The point  $a_1$  is fixed by the latent period of the reflex response to the first volley,  $9.8\sigma^*$  (average of 10 determinations). The line  $a_1v_1$  is drawn so that the slope corresponds to a velocity of propagation of 66 metres per second (average of 6 values). The central reflex-time  $d_1v_1$  thus calculated for the response to the first centripetal volley is  $2.9\sigma$ .

$a_2$  is fixed because the latent period of the response to the second centripetal volley was  $7.2\sigma$  (average of 4 values) when the  $C_1C_2$  interval was  $5.9\sigma$ . When

\* When the velocity of propagation in the afferent nerve fibres was being measured, a more powerful stimulus (16 cm. coil distance instead of 20 cm.) was applied by electrodes *c*, so as to make the reflex response of approximately the same size as that elicited from the root (see note \* on p. 515). The latent period was only reduced to  $9.65\sigma$  (average of 6 determinations), though the tension of the reflex response was increased from about 150 gm. to 600 gm.

$a_2v_2$  is drawn parallel to  $a_1v_1$ ,\* it is found that the central reflex-time  $d_2v_2$  is only  $0.3\sigma$ . The error involved in the measurement and calculation is not

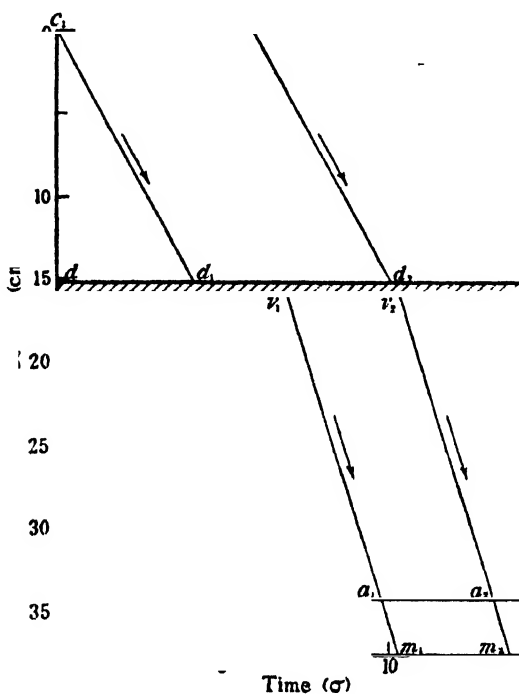


FIG. 4.

more than  $\pm 0.2\sigma$ , so it must be concluded that the shortest central reflex-time for the response to the second centripetal volley cannot be greater than  $0.5\sigma$ .

The observations in experiment 29.9.30 were almost as reliable as those just discussed and gave a value of  $0.4\sigma$  for the shortest central reflex-time of the response to the second centripetal volley, when the interval between the two volleys was  $5.9\sigma$ . In both these experiments the central reflex-time of the response to the second volley was found to be longer when the volley-interval was increased (see p. 527).

In all experiments (15) where there has been a short interval between two centripetal volleys, a similar shortening of the latent period of the second response has been observed, but in several the latent period of the reflex response was not measured. The values obtained in all those experiments

\* This is legitimate because experiment shows that the velocities of propagation of impulses in motor nerve fibres show no significant variation from fibre to fibre (cf. p. 518).

where such measurements were made are shown in Table III, columns 2 and 3.

Table III.

1	2	3	4
Date of experiment.	Shortest latent period of normal reflex (average values).	Shortest latent period of second response (average values).	Shortest conduction-time of peripheral path.
	$\sigma$	$\sigma$	$\sigma$
27.6.30	10.4	7.1	---
10.7.30	9.1	6.6	---
14.7.30	9.2	7.7	---
17.7.30	8.9	7.0	6.5
23.7.30	14.1	7.6	---
28.7.30	12.3	7.7	---
23.9.30	9.65	7.2	6.9
29.9.30	10.2	7.0	6.6

The conduction-time of the peripheral reflex path has only been measured in three of these experiments, but it has always lain between  $6\sigma$  and  $7\sigma$  when the electrodes have been applied to the afferent and efferent nerves at positions similar to those used in these experiments (*cf.* Table II). Since the values of column 3, Table III, lie between  $6.6\sigma$  and  $7.7\sigma$ , it is clear that all the other experiments support the evidence of experiments 23.9.30 and 29.9.30 in showing that the shortest central reflex-time of the response to  $C_2$ , when closely preceded by  $C_1$ , is very brief—probably not more than  $1\sigma$  for any experiment.

If the  $C_1C_2$  interval is increased, the shortening of latent period diminishes, until, at a sufficiently long  $C_1C_2$  interval, there is no significant shortening of latent period. Thus in Table IV there was a considerable shortening of latent period\* even when the stimulus-interval was as long as  $13.8\sigma$ .

Similar series of observations in five other experiments revealed similar shortenings of the latent period of the second response, and in all the shortening decreased as the  $C_1C_2$  interval was lengthened. The longest interval at which there was a shortening of latent period of more than  $1\sigma$  varied from  $7.9\sigma$  to  $32\sigma$  in different experiments.

(ii) *The Second Centripetal Volley in a different Afferent Nerve from the First.*—In these experiments the two afferent nerves have been either the two plantar nerves or the hamstring and popliteal nerves.

\* In this experiment the motor nerve was not stimulated, so it was only possible to measure the shortening of the latent period of the second response as compared with the first.

Table IV.

Stimulus-interval.	Response-interval measured for beginnings of action-currents.	Shortening of latent period.
$\sigma$	$\sigma$	$\sigma$
5.9	1.3	4.6
7.9	3.5	4.4
11.85	9.5	
	9.1	
	Average 9.3	2.55
	10.9	
13.8	13.0	
	Average 11.95	1.85
15.8	15.0	0.8
19.8	19.1	0.7
23.9	23.1	0.8
28.1	28.1	0.0
36.4	35.9	0.5

When the  $C_1C_2$  interval was 6  $\sigma$  or more, the shortening of the latent period of the response to  $C_2$  occurred exactly as if both  $C_1$  and  $C_2$  were set up in the same afferent nerve, and the central reflex-time was not more than 0.5  $\sigma$  under conditions of maximum shortening. As the  $C_1C_2$  interval was increased, the shortening was observed to diminish, until at intervals of 20  $\sigma$  or more it was usually insignificant. With  $C_1C_2$  intervals less than 6  $\sigma$  the shortening of the latent period of the response to  $C_2$  was less than with certain longer intervals, and, when both volleys were set up simultaneously, there was comparatively little shortening—seldom more than 1  $\sigma$ . This observation is in agreement with those previous experiments which showed that an increase in the size of a centripetal volley resulted in only a small reduction of the latent period as compared with the effect of one centripetal volley on another which followed it at a short interval (see note \*, p. 524).

#### IV. DISCUSSION.

From the considerations involved in the construction of fig. 4 (see p. 524) it is clear that the shortening of latent period cannot be due to time saved in the peripheral reflex paths. There must be a shortening of the central reflex-time. Moreover the same arguments applied to the central part of the reflex arc, show that the saving of time cannot occur at the expense of any time occupied in the actual propagation of impulses. At some central part of the

reflex arc there must be a place where a nerve impulse gives rise to a condition which endures for some time before setting up an impulse in the next part of the central pathway, *i.e.*, there is normally a delay at that point.

Thus these experiments support the conclusions drawn from other experimental observations (Eccles and Sherrington, 1930, *a*) that, in the flexor reflex, centripetal nerve impulses are not transmitted straight through the spinal cord, but at a certain point they are transformed into an enduring excitatory condition, *c.e.s.*, which may in turn set up fresh nerve impulses—the reflex discharge. These transformation points may be assumed provisionally to be the synapses (Eccles and Sherrington, 1930, *a* ; 1931, *c*).

The great reduction of the central reflex-time which has been found for the second of two responses must be entirely at the expense of the delay which normally occurs at the “synapses.” Therefore the shortest conduction-time through the central reflex arc cannot be greater than the shortest central reflex-time found for the second of two responses (not more than  $0.5\sigma$  in our two most reliable experiments). Measurements show that the central reflex pathway (*cf.* p. 516) cannot be shorter than 6 mm., and, as conduction along the finer branches of the afferent fibres and the dendrites would be slower than in the peripheral nerve fibres, it is not likely that the central conduction-time would be less than  $0.2\sigma$ . “Synaptic” delay is responsible for all the central reflex-time which is not conduction-time.

Before going on to discuss the nature of this delay and of its shortening, it will be well to state briefly the conditions resulting from the first centripetal volley.

When two centripetal volleys are set up in quick succession in the same afferent nerve, it has been shown that most of the motoneurones responding to the second volley are not excited by either volley alone (Eccles and Sherrington, 1931, *a*). Thus such motoneurones are in the subliminal fringe produced by either volley, and their response is due to facilitation. When there is a longer interval between two centripetal volleys, some motoneurones respond to both. Now from experimental observations the inference has been drawn that central excitatory state (*c.e.s.*) is greatly reduced by a reflex discharge from a motoneurone (Eccles and Sherrington, 1931, *b*) ; nevertheless impulses from a second centripetal volley will be likely to find preformed *c.e.s.* in a motoneurone which has responded to the previous volley, because *c.e.s.* would be produced by any delayed impulses of the first volley incident on the motoneurone after its first discharge. Thus the impulses of the second centripetal volley will find preformed *c.e.s.* both in the motoneurones which have responded

to the first volley and in those which have not. However, the former group of motoneurones will be relatively refractory for at least  $10\sigma$  after the first discharge (Eccles and Sherrington, 1931, *a*; see p. 553).

From experimental observations it has been inferred (Eccles and Sherrington, 1930, *a*) that several excitatory impulses, either in the same or in different afferent fibres, must be incident on a motoneurone before the resulting c.e.s. is sufficient to excite a reflex discharge. All the experimental observations on latent period can be simply explained, if it be assumed that, as the result of a single centripetal volley, the excitatory impulses incident on a motoneurone have a considerable temporal dispersion.\* The production of c.e.s. by each excitatory impulse is probably as brief as the action-current† of the impulse (not more than  $1\sigma$ ) but the c.e.s. produced by each impulse persists with gradually diminishing intensity for some time (Eccles, 1931, p. 580). Owing to the temporal dispersion of the incident excitatory impulses, the c.e.s. of a motoneurone would increase for a considerable time (*cf.* fig. 5), for it would be the aggregate of a series of superimposed step-like rises of c.e.s. produced by individual impulses. The rate of rise of the c.e.s. would depend on the rate of incidence of the excitatory impulses. The "synaptic" delay is the interval between the incidence of the first impulses on a motoneurone and the setting up of a reflex discharge. Since the reflex discharge is probably set up immediately the c.e.s. reaches neurone-threshold, the "synaptic" delay in any motoneurone will be the time necessary to build up c.e.s. to threshold value.

Fig. 5 is a diagrammatic representation of the c.e.s. of a motoneurone plotted as ordinates against time as abscissæ.  $OT_3$  shows time-curve of the c.e.s. produced by a single centripetal volley. If  $OT$  is the threshold value of c.e.s.,

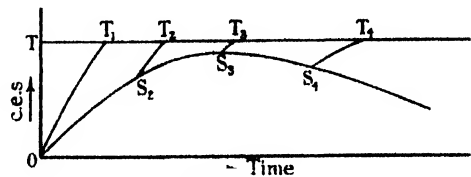


FIG. 6.

\* A similar assumption has also been found necessary in order to explain a number of other experimental results (*cf.* Eccles and Sherrington, 1931, *b*; 1931, *c*; Eccles, 1931). Part of the temporal dispersion would be due to differences in the conduction-times in the peripheral afferent path. This would arise from differences in the conduction velocities of impulses in different nerve fibres (Erlanger and Gasser, 1924).

† Negative after-potential is not taken account of in this connection (*vide* Eccles and Sherrington, 1931, *c*, p. 603).



$TT_2$  is the "synaptic" delay. Using conceptions of this type it is possible to explain the results of sections 4, 5 and 6 as follows:—

*Section 4.*—The rate of incidence of excitatory impulses on a motoneurone is higher with a large than with a small centripetal volley, so the resulting rise of c.e.s. is more rapid in the former case ( $OT_1$  in fig. 5) than in the latter case ( $OT_2$  in fig. 5). The synaptic delay for the larger volley,  $TT_1$ , is shorter than for the smaller volley,  $TT_2$ ; but a great reduction is not possible, for the additional nerve impulses set up by the stronger stimulus travel more slowly than those originally excited (since they are in nerve fibres of higher threshold), and so will reach the motoneurone relatively late. If  $S_1$  in fig. 5 is the time of the earliest arrival of such impulses,  $OS_1T_2$  and not  $OT_1$  would be the correct representation of the rise of c.e.s. produced by the larger volley, and  $TT_2$  would be the "synaptic" delay under those circumstances. From the diagram it is easy to see that a certain increase in a centripetal volley produces a much greater shortening of latent period when the volley is small than when it is large (*cf.* fig. 2). This explanation of the results of Section 4 accords with the former of the two alternatives mentioned on p. 518.

*Section 5.*—The c.e.s. produced by a centripetal volley will reach neurone-threshold at different times in different motoneurones probably in accordance with the respective rates of incidence of the excitatory impulses; hence there will be a temporal dispersion of the reflex discharges from the motoneurones, and this will appear in the action-current of the reflex response of the muscle.

*Section 6.*—In fig. 6  $OS_2S_3S_4$  represents the course of the subliminal c.e.s. produced in a motoneurone by the first centripetal volley. When the foremost impulses of a second similar centripetal volley are incident at  $S_3$ , it is clear that the "synaptic" delay (interval between  $S_3$  and  $T_3$ ) is shortest, and in motoneurones where the c.e.s. produced by the first volley just fails to reach neurone-threshold the "synaptic" delay would be still shorter. Thus the shortest central reflex-time of the second response would almost entirely be a central conduction-time. At longer intervals between the volleys, the "synaptic" delay of the second response would be longer in all motoneurones, *e.g.*, the interval between  $S_4$  and  $T_4$  in fig. 6. In a similar manner it is possible to explain the results which are obtained when the centripetal volleys are set up in different afferent nerves. At intervals such as  $OS_3$  or  $OS_4$  these results are identical with those already discussed. When the interval is shorter, *e.g.*,  $OS_2$ , it is clear that the "synaptic" delay at  $S_2$  ( $S_2T_2$  interval in fig. 6) is longer than the "synaptic" delay at  $S_3$  ( $S_3T_3$  interval in fig. 6). Finally, when both volleys are simultaneous, the "synaptic" delay ( $TT_1$  in fig. 6) is

still longer (*cf.* observations on p. 527). It is clear from fig. 6 that the interval between the volleys at which there is the shortest "synaptic" delay corresponds to the interval between the beginning of the c.e.s. production and the attainment of a maximum development of c.e.s. in the average motoneurone. Values of  $6\sigma$  to  $8\sigma$  are usual for this interval in experiments where the centripetal volleys are set up in different afferent nerves.

In explaining the results of section 6 attention has so far been confined to those motoneurones in which the first centripetal volley produced a subliminal c.e.s. When the interval between the volleys was short, it has been shown (Eccles and Sherrington, 1931, *a*) that the response to the second volley was almost entirely due to facilitation of such motoneurones of the subliminal fringe. Of course, when two centripetal volleys are set up at very short intervals, the impulses from the second will be incident on some motoneurones before these particular motoneurones respond to the first. The foremost impulses of the second volley arrive before some of the delayed impulses of the first. Under these circumstances the "synaptic" delay of the response of such motoneurones to the first volley is shortened by impulses of the second volley. Such a temporal overlap of the effects of two volleys must be usual at short intervals, if the impulses of one volley have a temporal dispersion as great as that indicated in the preceding discussion.

With longer intervals between the centripetal volleys, the impulses of the second volley would be incident on motoneurones during the refractory period following their response to the first volley (Eccles and Sherrington, 1931, *a*), and so a reflex discharge, if evoked from them, would not have a short "synaptic" delay, even though there was some augmentation of the effects of the second volley by delayed impulses of the first volley (*cf.* p. 528). Finally, at still longer intervals between the two volleys, the effects of the refractory period will have passed off, and any c.e.s. remaining from the effects of the first volley will cause the second response to have a shorter "synaptic" delay than normal.

In some experiments where the facilitated response has had a greatly shortened latent period, the electrical record of the response has shown a second distinct action-current with a latent period similar to that of a normal reflex. This can be clearly seen in the lower record in Plate 36, fig. 7. Following the large motor action-current, there is first the action-current of the facilitated response partly obscured by the myograph line, and then the smaller second action-current. The first has a "modal" latent period of  $8.3\sigma$ , the second  $14.3\sigma$ . Immediately above is a record where the second centripetal volley was not

preceded by the first. The "modal" latent period of  $14.6\sigma$  under this latter condition agrees well with the value of  $14.3\sigma$  for the latent period of the second action-current in the other record. This second action-current seems to be due to the discharge of motoneurones which also responded to the first volley, the second response being delayed by the refractory period following the first discharge.

The adequate explanation afforded for all the above experimental observations by temporal dispersion of the incident excitatory impulses argues in justification of the correctness of this assumption, especially as a similar assumption has afforded a satisfactory explanation of other experimental observations (Eccles and Sherrington, 1931, *b*; 1931, *c*, p. 599; Eccles, 1931).

Since the shortening of latent period of the second response has been observed in all experiments where the second response was not greatly inhibited by the first centripetal volley (Eccles and Sherrington, 1931, *a*), our results are difficult to reconcile with those of Adrian and Olmsted (1922) and with those of Forbes, Querido, Whitaker, and Hurxthal (1928). When there was a short interval ( $20\sigma$ ) between two stimuli applied to an afferent nerve, the former observers found, in three experiments, that the second reflex response had a latent period  $2.8\sigma$  to  $4.8\sigma$  *longer* than the first. Forbes and co-workers (1928) record maximal *lengthenings* of  $2.5\sigma$  and  $3.5\sigma$  in two experiments, but, in those experiments where the second response was large, a slight shortening was observed. Usually the changes were almost within the limits of experimental error. It is possible in some cases at least that the action-current of the early facilitated response, which is often superimposed on the response to the first centripetal volley, was unnoticed, and only the latent period of a second action-current such as that in Plate 36, fig. 7, was measured.

#### SUMMARY.

A method for measuring the latent period of the flexor reflex is described. The velocity of propagation of impulses in both the afferent and efferent nerve fibres is also measured to enable calculation of the central reflex-time. The values obtained for this range from  $2.75\sigma$  to  $4.35\sigma$ , and are in general agreement with the values calculated by Jolly and by Forbes and Gregg.

The central reflex-time is found to be shortened when the stimulus applied to the afferent nerve is strengthened.

The temporal dispersion of many reflex discharges is shown to be due, not to the discharge of more than one impulse from motoneurones, but to variations in the latent periods of the single responses of different motoneurones.

The latent period of the response to a centripetal volley is greatly shortened if another volley precedes it by certain intervals. This shortening of latent period occurs at the expense of the central reflex-time which in some cases is reduced to less than  $0.5\sigma$ . It is concluded that all the time is saved in the reduction of the normal "synaptic" delay by facilitation. If that is so, the actual conduction-time through the spinal cord must be less than  $0.5\sigma$ . On the assumption that the normal "synaptic" delay is due to time taken for a succession of excitatory impulses (owing to their temporal dispersion) to build up a c.e.s. of threshold intensity, all the observations are satisfactorily explained.

The experiments support the conclusions drawn from other experimental observations that, in the flexor reflex, centripetal impulses are not transmitted straight through the spinal cord, but at certain points ("synapses") they are transformed into an enduring excitatory condition, c.e.s., which may in turn set up fresh nerve impulses—the reflex discharge.

One of us (J.C.E.) wishes to express his gratitude to the Medical Research Council for a personal grant, and we have to thank the Royal Society and the Rockefeller Foundation for grants towards the apparatus used in this research.

#### REFERENCES.

- Adrian, E. D., and Bronk, D. W. (1929). 'J. Physiol.,' vol. 67, p. 119.  
Adrian, E. D., and Forbes, A. (1922). 'J. Physiol.,' vol. 56, p. 301.  
Adrian, E. D., and Olmsted, J. M. D. (1922). 'J. Physiol.,' vol. 56, p. 426.  
Buchanan, F. (1907). 'Proc. Roy. Soc.,' vol. 79, p. 503.  
Buchanan, F. (1908). 'Quart. J. Exp. Physiol.,' vol. 1, p. 1.  
Cooper, S., and Eccles, J. C. (1930). 'Proc. Physiol. Soc.,' p. iii; 'J. Physiol.,' vol. 69.  
Eccles, J. C. (1931). 'Proc. Roy. Soc.,' B, vol. 107, p. 557.  
Eccles, J. C., and Sherrington, C. S. (1930, a). 'J. Physiol.,' vol. 69, p. 1.  
Eccles, J. C., and Sherrington, C. S. (1930, b). 'Proc. Physiol. Soc.,' p. i; 'J. Physiol.,' vol. 69.  
Eccles, J. C., and Sherrington, C. S. (1931, a). 'Proc. Roy. Soc.,' B, vol. 107, p. 535.  
Eccles, J. C., and Sherrington, C. S. (1931, b). 'Proc. Roy. Soc.,' B, vol. 107, p. 586.  
Eccles, J. C., and Sherrington, C. S. (1931, c). 'Proc. Roy. Soc.,' B, vol. 107, p. 597.  
Erlanger, J., Bishop, G. H., and Gasser, H. S. (1926). 'Amer. J. Physiol.,' vol. 78, p. 574.  
Erlanger, J., and Gasser, H. S. (1924). 'Amer. J. Physiol.,' vol. 70, p. 624.  
Erlanger, J., and Gasser, H. S. (1930). 'Amer. J. Physiol.,' vol. 92, p. 43.  
Forbes, A., and Gregg, A. (1915). 'Amer. J. Physiol.,' vol. 37, p. 118.  
Forbes, A., Querido, A., Whitaker, L. R., and Hurxthal, L. M. (1928). 'Amer. J. Physiol.,' vol. 85, p. 432.  
Gasser, H. S., and Erlanger, J. (1930). 'Amer. J. Physiol.,' vol. 94, p. 247.  
Jolly, W. A. (1911). 'Quart. J. Exp. Physiol.,' vol. 4, p. 67.  
Lenninger, E. (1913). 'Z. Biol.,' vol. 60, p. 75.

- Sherrington, C. S. (1906). "The Integrative Action of the Nervous System," New Haven.  
 Sherrington, C. S. (1921, a). 'Proc. Roy. Soc.,' B, vol. 92, p. 245.  
 Sherrington, C. S. (1921, b). 'Arch. Internat. Physiol.' vol. 18, p. 620.

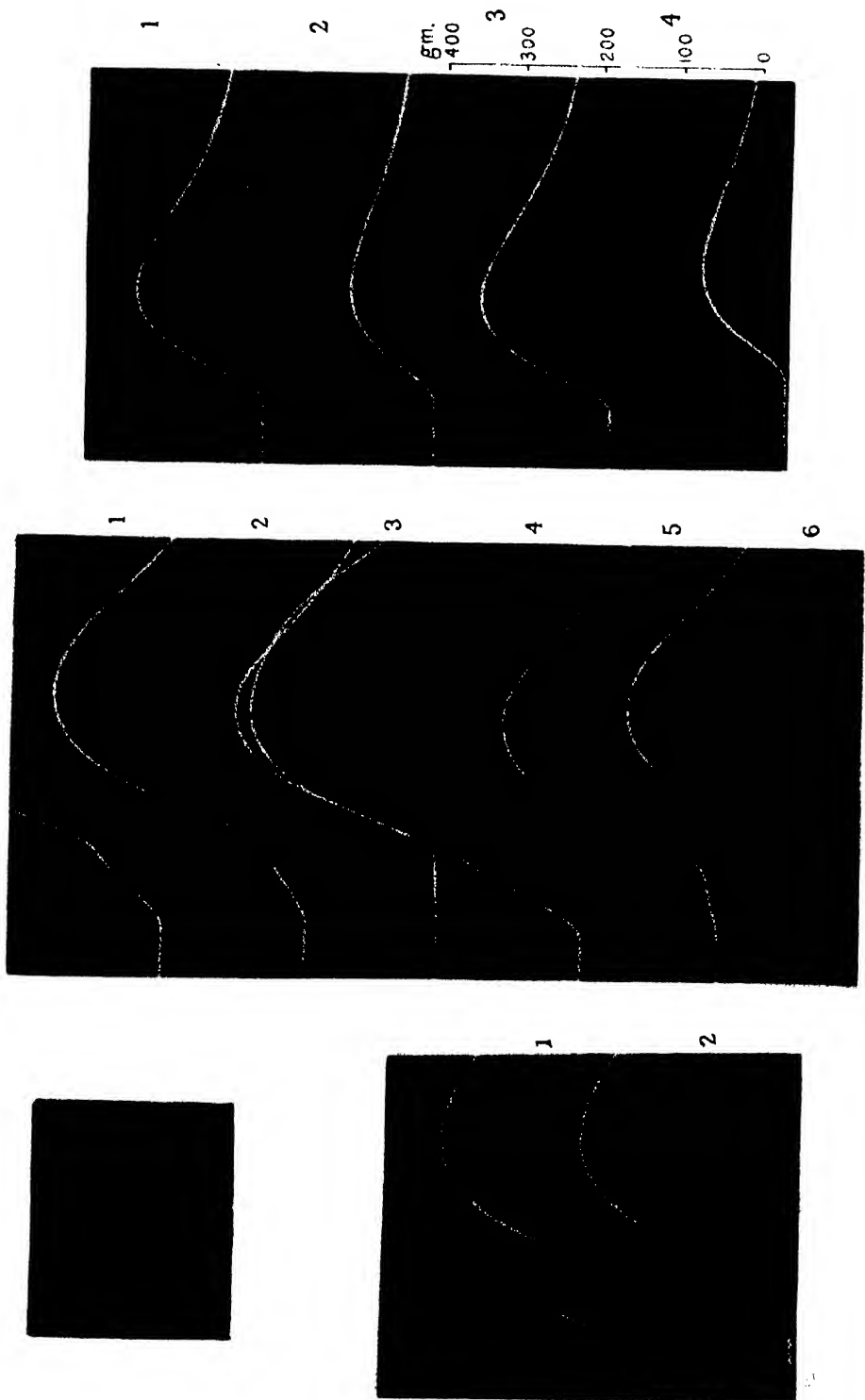
## EXPLANATION OF PLATES.

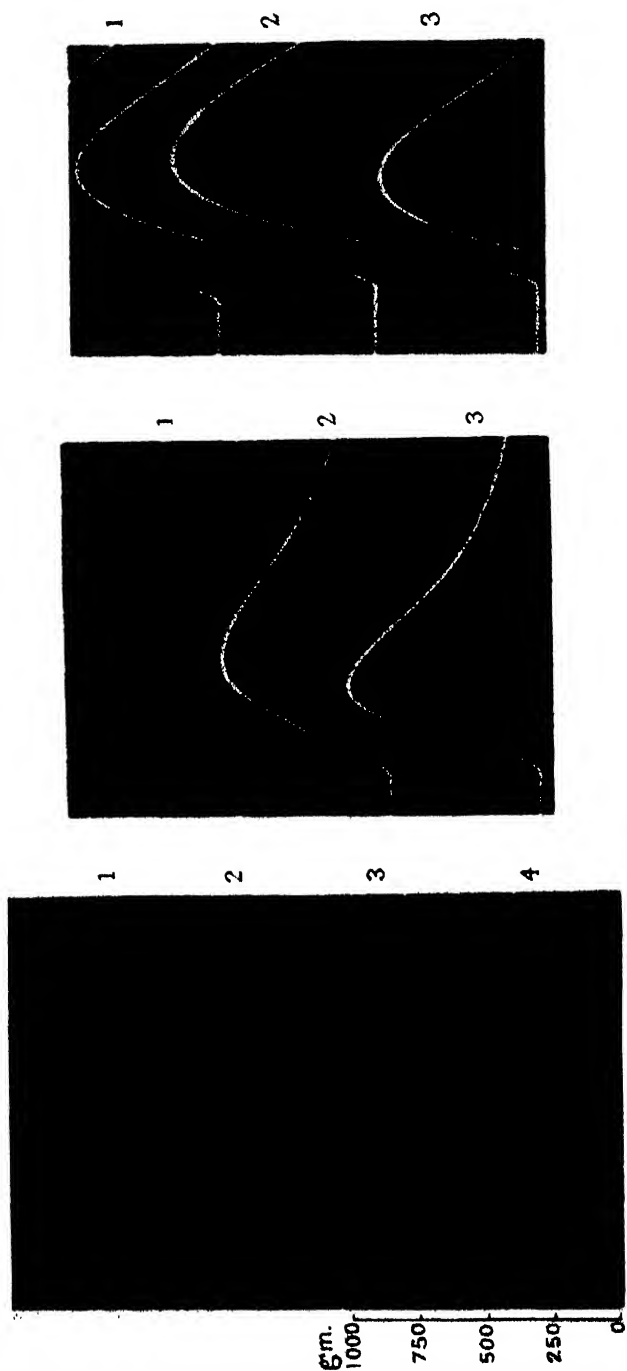
## PLATE 35.

- FIG. 1.—Movement of string resulting from the instantaneous break of a current of  $3 \times 10^{-4}$  amps. flowing through it.  
 FIG. 2.—Electrical responses of tibialis anticus muscle showing reflex responses followed by maximal motor responses. The reflex is elicited in the upper record by a stimulus applied to popliteal nerve in the middle of the thigh, and in the lower record by a stimulus applied to the 8th post-thoracic dorsal root. Time, 1 d.v. = 10  $\sigma$ . The white lines show part of the myographic record.  
 FIG. 3.—A series of records showing reflex responses of tibialis anticus muscle followed by maximal motor responses. The strength of the stimulus (applied to popliteal nerve) eliciting the reflex was varied. The white lines of the myographic record commence below the corresponding electrical record. Time, 1 d.v. = 10  $\sigma$ . Tension scale as for Plate 36, fig. 5.  
 FIG. 4.—Reflex responses of tibialis anticus muscle evoked by one or two centripetal volleys in popliteal nerve as follows:—1,  $C_1$  5.9  $\sigma$   $C_2$ . 2,  $C_1$  alone. 3,  $C_1$  4.0  $\sigma$   $C_2$ . 4,  $C_2$  alone. Time, 1 d.v. = 10  $\sigma$ . Tension scale at side.

## PLATE 36.

- FIG. 5.—Reflex responses of tibialis anticus muscle evoked by one or two centripetal volleys in popliteal nerve followed by maximal motor responses:—1,  $C_2$  followed by motor response. 2,  $C_1$  4  $\sigma$   $C_2$  followed by motor response. 3,  $C_1$  5.9  $\sigma$   $C_2$  followed by motor response. 4,  $C_1$  7.9  $\sigma$   $C_2$  followed by motor response. In observation 3 it is possible to recognise the action-current of the reflex evoked by  $C_2$  beginning at the summit of the response to  $C_1$ . The action-current of the response to  $C_1$  alone is shown by the first part of observation 4. Time, 1 d.v. = 10  $\sigma$ . Tension scale at side.  
 FIG. 6.—Reflex responses of tibialis anticus muscle evoked by two centripetal volleys in popliteal nerve at the following intervals:—1,  $C_1$  32.2  $\sigma$   $C_2$ . 2,  $C_1$  19.8  $\sigma$   $C_2$ . 3,  $C_1$  5.9  $\sigma$   $C_2$ . In observation 3 it is possible to recognise the action-current of the reflex evoked by  $C_2$  beginning just before the summit of the response to  $C_1$ . The action-current of the response to  $C_1$  alone is shown by the first parts of observations 1 and 2. Time, 1 d.v. = 10  $\sigma$ . Tension scale as for Plate 35, fig. 4.  
 FIG. 7.—Responses of tibialis anticus muscle evoked as follows:—1, A alone. 2, A 4.8  $\sigma$   $C_2$ . 3,  $C_1$  1.6  $\sigma$  A 4.8  $\sigma$   $C_2$ . A is the stimulus to the motor nerve (maximal).  $C_1$  and  $C_2$  are the first and second stimuli to the popliteal nerve (evoking reflex responses). In observation 3 there is seen to be a large reflex action-current immediately following the maximal motor response (shown by observation 1) and this in turn is followed by a smaller action current with a latent period similar to that of the response in observation 2. Time, 1 d.v. = 10  $\sigma$ .





*Studies on the Flexor Reflex.—II. The Reflex Response Evoked by Two Centripetal Volleys.*

By J. C. ECCLES, M.A., D.Phil., Research Fellow of Exeter College, and  
Sir Charles S. SHERRINGTON, O.M., F.R.S., Physiological Laboratory,  
Oxford.

(Received January 2, 1931.)

[PLATES 37, 38.]

CONTENTS.

	PAGE
I. Introduction .....	535
II. Method .....	536
III. Results .....	537
1. The reflex response evoked by the second centripetal volley does not suffer any appreciable diminution by the first .....	537
i. Experimental observations .....	537
ii. Discussion .....	543
2. The reflex response evoked by the second centripetal volley is diminished by the first, but only at certain intervals less than $50\sigma$ .....	545
3. The reflex response evoked by the second centripetal volley is diminished by the first, even at intervals longer than $80\sigma$ .....	547
IV. Discussion .....	550

I. INTRODUCTION.

When an afferent nerve is stimulated by two similar break-shocks at a sufficiently short interval, it has been found that the ensuing reflex response is no greater than the response to a single break-shock. The shortest interval at which the second stimulus produces an additional response has been called "the least interval for muscular summation" (Adrian and Olmsted, 1922). Different observers have found fairly comparable values for this interval in the mammalian preparation:  $0.75\sigma$  to  $1.31\sigma$  (Sherrington and Sowton, 1915);  $1.2\sigma$  to  $2.4\sigma$  (Adrian and Olmsted, 1922);  $1.0\sigma$  to  $2.4\sigma$  (Forbes, Querido, Whitaker and Hurxthal, 1928).

It was at first thought that the ineffectiveness of the second stimulus at intervals less than "the least interval" was due to a refractory period of the central part of the reflex arc; consequently the "least interval" was used as



a measure of the duration of the absolutely refractory period of the reflex arc. Forbes (1922), however, pointed out that the duration of the "least interval" was similar to the duration of the absolutely refractory period of the afferent nerve (*cf.* Sherrington, 1921, p. 257), and concluded that the ineffectiveness of the second stimulus was probably due to its inability to set up centripetal impulses in the afferent nerve, and not to a blocking of such impulses by a refractory period in some part of the central reflex pathway. Later (1928) Forbes and his co-workers suggested that the reflex response added by the second centripetal volley, when it followed the first at very short intervals, might not be due to a second discharge of those motoneurons which had already responded to the first volley. Other motoneurons might be excited by summation of the central effects of the two centripetal volleys. If that were so, "the least interval for muscular summation" would have no relation to the refractory period of the reflex pathway.

Bremer (1930, *b*) arrived at a similar conclusion and from the "curve of summation" (constructed from the amount of reflex contraction added by a second centripetal volley to that evoked by the first) he calculated that in the frog at 18° C. the absolutely refractory period of the "synaptic" region of the reflex arc had a duration between 7  $\sigma$  and 15  $\sigma$ , most probably a little less than 10  $\sigma$ .

During the investigation of the reflex responses (in the efferent nerve trunk) evoked by two centripetal volleys at various intervals, Forbes and his co-workers (1928) noticed that in some experiments the second response was increased by the first and in other experiments it was diminished even when the interval was as long as 50  $\sigma$ . The former effect was attributed to facilitation, the latter was reinvestigated by Gerard and Forbes (1928) who came to the conclusion that it was a phenomenon analogous to equilibration in peripheral nerve. They found that it may last 0.8 second or even longer.

In the following investigation new methods of analysis have been employed in studying the reflex response evoked by two centripetal volleys at various intervals. A preliminary account of some of the observations has been communicated to the Physiological Society (Eccles and Sherrington, 1930, *c*).

## II. METHOD.

The general experimental procedure was similar to that described in the previous paper (Eccles and Sherrington, 1931, *a*).

The muscle (tibialis anticus) was completely deafferented in all experiments in which an antidromic volley was set up, and in almost all of the other experiments. In the two

experiments in which the deafferentation was performed after some of the observations had been taken, no resulting alteration of the type of the responses could be detected.

### III. RESULTS.

According to the effect which one centripetal volley ( $C_1$ ) exerts on the reflex response evoked by a second centripetal volley ( $C_2$ ), it is possible to divide the experimental results into three types. These types will be discussed separately, though the divisions between them are not precise and transitions often occur.

#### 1. *The Reflex Response Evoked by the Second Centripetal Volley does not suffer appreciable Diminution by the First.*

Series of observations conforming to this type were comparatively rare, being only obtained in seven out of a total of over forty experiments.

(i) *Experimental Observations.*—In the series shown in Plate 37, fig. 1, it is apparent from the action-currents that the reflex response evoked by  $C_2$  was very little affected by the precedence of  $C_1$  even at an interval as short as  $15.8\sigma$ .  $C_2$  was weaker than  $C_1$ , and, since both were set up by break-shocks through the same electrodes, all the motoneurones responding to  $C_2$  also had responded to  $C_1$ . The size of a reflex response is, however, not reliably indicated by its action-current, for this is affected by the degree of temporal dispersion of the reflex (Forbes and Gregg, 1915; Eccles and Sherrington, 1931, a). Analysis of the myogram was, therefore, employed in order to obtain a measure of the second reflex response. The method used (called the method of maximum tension differences) has been checked by working with maximal and submaximal motor twitches, and has been found sufficiently accurate for dealing with records of reflex responses\* (Eccles and Hoff, 1931).

The ordinates of the series of observations plotted in fig. 1 are the tensions (calculated by the above method) of the reflex responses evoked by  $C_2$  at the  $C_1C_2$  intervals indicated by the abscissæ. It will be seen that for  $C_1C_2$  intervals longer than  $16\sigma$  the tension of the response calculated for  $C_2$  is about 1.5 times that of the response to  $C_2$  alone, and that there is a slight progressive decline as the  $C_1C_2$  interval lengthens. Since both these features arise from the method employed in the analysis of the combined responses (*cf.* note below),

\* This method is not strictly applicable to such series of observations, for some of the motor units excited in the second reflex response may have also responded in the first reflex. The tension calculated for these motor units will usually be about 1.3 to 1.5 times their normal tension (Eccles and Hoff, 1931). The tension calculated for the other motor units will have suffered no such increase.

it may be concluded that at these intervals the reflex response evoked by  $C_2$  is unaffected by the precedence of  $C_1$ . At shorter intervals there is a

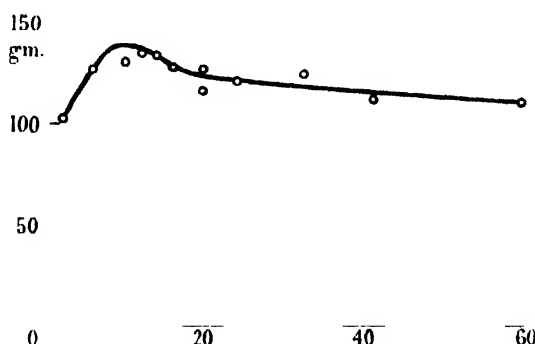


FIG. 1.—Maximum tension of the reflex response evoked by  $C_2$  (ordinates) plotted against the intervals in sigma by which it follows  $C_1$  (abscissae). The tensions of reflexes evoked by  $C_2$  alone are shown by the two arrows.

slight increase of the tension of the response to  $C_2$  which cannot be accounted for as the result of the method of analysis, and at a  $C_1C_2$  interval of  $4\sigma$  the reflex evoked by  $C_2$  is less than normal.

In three experiments analysis of the myogram showed a much greater increase of tension of the response to  $C_2$  and this was obtained over a longer range of  $C_1C_2$  intervals. Thus in Plate 37, fig. 2, both the electrical and mechanical responses show that, at  $C_1C_2$  intervals of  $20\sigma$  or less, the reflex response evoked by  $C_2$  was much greater than normal. This is confirmed when the tensions (calculated as above) of the responses to  $C_2$  in all the observations of this series are plotted against the corresponding  $C_1C_2$  intervals (fig. 2, cf. fig. 1). The large increase of response to  $C_2$  when the  $C_1C_2$  interval was  $20\sigma$  or less must have been due to a reflex discharge from many motoneurones which were not excited to discharge by  $C_2$  alone. Similar series of observations have been obtained in two other experiments, *e.g.*, Plate 37, fig. 3, and comparable results have been described by Forbes and co-workers (1928), and by Bremer (1930, a).

When the two centripetal volleys  $C_1$  and  $C_2$  were so small that neither alone evoked a reflex response, it has been found that a response was produced when one followed the other at a suitable interval (Sherrington, 1929, p. 343; Bremer, 1930, a; Eccles and Sherrington, 1930, a). It has been argued that the first volley must set up in some motoneurones an enduring excitatory process which sums with a similar process produced by the second volley to give rise to an intensity of excitation sufficient to evoke a reflex discharge

from those motoneurons. This enduring excitatory process has been called the central excitatory state (c.e.s.).

When both  $C_1$  and  $C_2$  are strengthened so that either alone evokes a reflex discharge from some motoneurons, the observations just described show that a similar process of summation of c.e.s. must be causing other motoneurons to discharge reflexly at certain  $C_1C_2$  intervals. These latter motoneurons must be subliminally excited by both  $C_1$  and  $C_2$  alone, *i.e.*, they belong to the subliminal fringes produced by both  $C_1$  and  $C_2$  (Denny-Brown and Sherrington, 1928; Sherrington, 1929).

The comparatively small responses\* evoked by  $C_2$  at long  $C_1C_2$  intervals, *e.g.*, at more than 30  $\sigma$  intervals in Plate 37, fig. 2, and text-fig. 2, were most probably the result of a reflex discharge from the motoneurons which responded when  $C_2$  alone was employed as a stimulus, and these motoneurons would be among those which responded to  $C_1$  alone (see p. 537). A similar conclusion has already been drawn for the responses in Plate 37, fig. 1, and text-fig. 1, when the  $C_1C_2$  interval was 16  $\sigma$  or more.

Though the size of the combined reflex response at short  $C_1C_2$  intervals in Plate 37, fig. 2, and text-fig. 2 is so great that many motor units must have been excited by the process of facilitation described above, it is probable that some of the contraction added by  $C_2$  was due to the excitation of motor units which had also responded to  $C_1$ . This can be investigated for very short  $C_1C_2$  intervals by the method of "backfiring" (Eccles, 1931) which has already been used to test whether the reflex response evoked by a single centripetal volley was repetitive or not (Eccles and Sherrington, 1931, *a*, p. 519). The procedure is best described with reference to a diagrammatic representation of a particular experiment. The construction of this diagram is similar to that described in the previous paper (Eccles and Sherrington, 1931, *a*, p. 520).

The conditions of experiment 19.6.30 are shown in fig. 3. The sizes of the

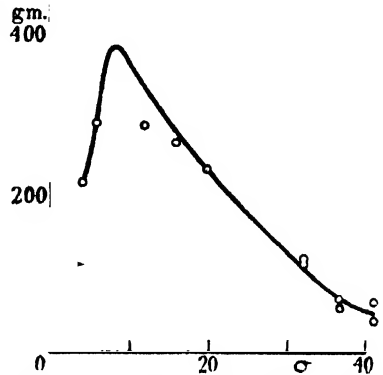


FIG. 2.—As in fig. 2 in another experiment. The tension of the reflex evoked by  $C_2$  alone is shown by the arrow.

\* These responses were less than the response to  $C_2$  alone, because there was probably a weak inhibition of the motoneurons by some of the centripetal impulses in  $C_1$  (see p. 547; *cf.* Eccles, 1931, note, p. 572).



The motoneurones of the reflex centre can be divided into two groups according to whether they did or did not respond to  $c_1d_1$  alone. When  $c_2d_2$  followed  $c_1d_1$ ,  $v_3a_3m_2$  and  $v_2y^*$  represent the reflex discharges evoked by  $c_2d_2$  in the former and latter groups of motoneurones respectively. It can be seen that the discharge  $v_3a_3m_2$  would pass to the muscle exciting it to respond by a contraction which would be superposed on the maximal motor twitch evoked by  $a_1m_1$ . On the other hand,  $v_2y$  would be blocked by the antidromic volley  $a_1y$  at  $y$ , because in that group of motor nerve fibres the antidromic volley was not blocked by  $v_1x$ . Thus, in only those motor units which had already been excited by  $c_1d_1$ , was the reflex discharge evoked by  $c_2d_2$  able to reach the muscle and give rise to a contraction additional to the maximal motor twitch.† The antidromic volley would block the discharge from all those motoneurones which did not respond to  $c_1d_1$  alone, and consequently also to  $c_2d_2$  alone (for  $c_2d_2$  was adjusted so as to be identical with  $c_1d_1$ ), i.e., the reflex discharge from those motoneurones whose response was evoked by the facilitation of the effect of  $c_2d_2$  by  $c_1d_1$ .

In columns 5 and 6 of Table I the tension added to the maximal motor twitch by the reflex response is calculated by the method of maximum tension differences (Eccles and Hoff, 1931; see p. 537).

The results of experiment 19.6.30 are shown in Table I. In the first series the two centripetal volleys together gave rise to a reflex response producing 80 grm. (208–128) tension more than a single volley; but an antidromic volley blocked the reflex discharge from either one or both centripetal volleys (as shown by the absence of any appreciable addition of tension to the motor twitch). This is confirmed by the electrical record, which shows no action-currents additional to that of the motor twitch. There is a similar result in the second series of experiment 19.6.30 with two larger centripetal volleys. Again in experiment 9.7.30, 142 grm. (285–143) extra response was added by the second centripetal volley almost entirely by exciting fresh neurones. Four experiments not shown in Table I also gave similar results. In these observations almost all of the extra reflex response produced by the second centripetal

\* The central reflex time of  $v_2y$  would be shorter than that of  $v_3a_3m_2$  (Eccles and Sherrington, 1931, a).

† This conclusion is only legitimate when the  $c_1c_2$  interval was so short that there was no possibility of a reflex discharge being evoked by  $c_2d_2$  in some motoneurones after the passing off of the refractory period following the antidromic volley, e.g., at  $v_3$ . The maximal duration of the  $c_1c_2$  interval which can safely be used, depends on the temporal dispersion of the reflex discharge evoked by  $c_2d_2$ . With most reflexes 6  $\sigma$  is a safe maximum for the  $c_1c_2$  interval.

Table I.

1	2	3	4	5	6
Date of experiment.	Interval between the centripetal volleys.	Maximum tension of single reflex twitch.	Maximum tension of reflex response to the two centripetal volleys.	Maximum tension added reflexly by a single centripetal volley to the maximal motor twitch.	Maximum tension added by the two centripetal volleys to the maximal motor twitch.
	$\sigma$	gm.	gm.	gm.	gm.
19.6.30	2.8	123 132	202 213	8 -12	
		Average 128	Average 208	Average -2	0
19.6.30	2.8	191 202 220 229	290 302 — —	8 6 — —	0 8 — —
		Average 210	Average 296	Average 7	Average 4
19.6.30	2.8	311 325	393 —	50 -12	97 82
		Average 318	393	Average 19	Average 90
9.7.30	4.0	131 179 135 125	295 275 — —	0 -34 46 6	14 2 — —
		Average 143	Average 285	Average 3	Average 8
14.7.30	5.6	563 610	— —	17 14	185 91
		Average 587	754	Average 16	Average 138

volley must occur in motor units not excited by either volley alone, i.e., it is due to a discharge from motoneurons of the subliminal fringe.

When the centripetal volleys in experiment 19.6.30 were made still larger, the results in the third series of Table I show that almost all of the 75 gm. (393-318) added by the second volley to the reflex response evoked by the first volley, was not blocked by the antidromic volley. Therefore the tension added by the second volley was then produced by the excitation of motor-units which had already responded to the first volley.

Similar results were obtained in experiment 14.7.30. When the centripetal volleys were large 167 gm. (754-587) was added by the second centripetal volley to the reflex response of the first. This could be, for the most part,

accounted for by the excitation of motor-units which had already responded to the first volley (Table I). On the other hand, when the centripetal volleys were small, the reflex discharge from motoneurons of the subliminal fringe was almost entirely responsible for the extra response evoked by the second volley when it followed the first (not shown in Table I).

In some experiments it could not be shown that two centripetal volleys in quick succession excited some motoneurons to respond twice. When the centripetal volleys were made sufficiently large, one volley alone was found to produce a reflex response with so much after-discharge that the effect of two volleys could not be analysed.

(ii) *Discussion*.—Thus when they are large, centripetal volleys in quick succession may excite some motoneurons to respond twice; when they are small, the second volley is ineffective. The first centripetal volley must be followed by a period of unresponsiveness\* in the discharging motoneurons. In two experiments it has been shown that after a certain interval the unresponsiveness was relative. In other experiments this could not be done, probably because it was not possible to make the observations at a sufficiently long time after the first centripetal volley (see note †, p. 541).

Though in experiment 19.6.30 motoneurons responded to both  $C_1$  and  $C_2$  when the  $C_1C_2$  interval was only  $2.8\sigma$ , the second discharge of these motoneurons probably took place more than  $2.8\sigma$  after the first discharge (*cf.* Adrian and Olmsted, 1922). Therefore it is not justifiable to conclude that the period of absolute unresponsiveness was shorter than  $2.8\sigma$  in this experiment.

The time necessary for complete recovery from the period of unresponsiveness is difficult to estimate owing to masking by the discharge of the facilitated motoneurons. In those experiments where facilitation was only present at relatively short intervals, it is possible to place a significant upper limit to the duration of the period of relative unresponsiveness. Thus in Plate 37, fig. 1, the response to  $C_2$  seems to be unaffected by the precedence of  $C_1$  even at an interval of  $15.8\sigma$ . At shorter intervals, *e.g.*, at  $7.9\sigma$  or  $5.9\sigma$  the accompanying facilitation prevents any estimation of the degree of recovery of those motoneurons which had already responded to  $C_1$ . In that experiment  $15.8\sigma$  would therefore be an upper limit to the duration of the period of relative

\* This period of unresponsiveness, absolute or relative, may be a refractory period, but it will be seen later that some types of unresponsiveness following a centripetal volley are due to inhibition.



unresponsiveness following  $C_1$ . The observations from which fig. 1 was constructed also indicate a similar upper limit.

With  $C_1C_2$  intervals less than this upper limit the motoneurones responding to  $C_2$  are probably partly those normally excited by  $C_2$ , partly those motoneurones of the subliminal fringe which are facilitated. The shorter the interval the more the latter group predominates, until finally it is found that, at very short  $C_1C_2$  intervals, the former group becomes negligible owing to the period of unresponsiveness following  $C_1$ . The nature of this period of unresponsiveness will be considered later (see p. 550).

It has been shown that most of the reflex response evoked by  $C_2$  at very short  $C_1C_2$  intervals was due to the facilitation of motoneurones of the subliminal fringe. Assuming an average value for the tension of an individual motor unit (Eccles and Sherrington, 1930, *b*), it is possible to calculate the percentage of motoneurones that can be shown to be in the subliminal fringe for any particular strength of stimulus. Thus in experiment 19.6.30 when the stimuli applied to the afferent nerve were weak, 13.8 per cent. of the total motor-units were excited by a single centripetal volley, and a further 8.7 per cent. were in the subliminal fringe. On strengthening the stimulus the numbers were 22.9 per cent. and 9.2 per cent. respectively. The number of motoneurones excited by a single volley was augmented, presumably by increased excitation of the motoneurones of the subliminal fringe. Fresh motoneurones must, however, have been added to the subliminal fringe, for it suffered no reduction. When  $C_1$  and  $C_2$  were made still larger, the number of motoneurones excited by a single centripetal volley was increased to 34.4 per cent., but the subliminal fringe had almost disappeared. In experiment 9.7.30 the subliminal fringe was larger, the respective values being 14.0 per cent. and 13.8 per cent.

The values given for the subliminal fringe will be too low in all these examples, because the second stimulus was applied to the afferent nerve during the persistence in the afferent nerve fibres of the relatively refractory period following the first stimulus (Adrian and Forbes, 1922). Under these circumstances the second centripetal volley would not be so powerful as it would be in the normal nerve, for impulses would be set up in fewer fibres and each impulse would be subnormal in size. However, in addition to this consideration, two similar centripetal volleys can only evoke a reflex discharge in those motoneurones in which each produces not less than half the threshold value of c.e.s. (assuming that c.e.s. can be simply summed). Those motoneurones of the subliminal fringe which can be excited to discharge by two similar centripetal volleys belong to the "effective subliminal fringe."

2. *The Reflex Response Evoked by the Second Centripetal Volley is Diminished by the First, but only at certain Intervals less than 50  $\sigma$ .*

Series of observations conforming to this type have been obtained in 10 experiments (*cf.* Forbes and co-workers, 1928, fig. 2, observations B and E).

Part of such a series is shown in Plate 38, fig. 4. It is clear from both the electrical and mechanical responses that, when the  $C_1C_2$  interval was 13.8  $\sigma$ , the response to  $C_2$  was greatly reduced, whereas when the  $C_1C_2$  interval was 41  $\sigma$ , the response evoked by  $C_2$  was unaffected by  $C_1$ . Also there seems very little effect on  $C_2$  when the interval was 19.8  $\sigma$ . The whole series of the responses to  $C_2$  (calculated as on p. 537) are plotted against the  $C_1C_2$  intervals in fig. 4. The response to  $C_2$  was probably normal when the  $C_1C_2$  interval was 28  $\sigma$  or more, while at a  $C_1C_2$  interval of 14  $\sigma$  there seems to be a "minimum." The period necessary for complete recovery from the effect of  $C_1$  varied from 20  $\sigma$  to 50  $\sigma$  in different experiments, and in most there was a definite "minimum" at 10  $\sigma$  to 20  $\sigma$  after  $C_1$ .

By means of a suitably timed antidromic volley it was possible to show that at short  $C_1C_2$  intervals the response to  $C_2$  was due solely to the facilitation of the motoneurons of the subliminal fringe (*see* p. 541). It therefore seems likely (on analogy with the results of the previous section, p. 539) that some of the response to  $C_2$  at longer intervals was also due to facilitation. The amount of

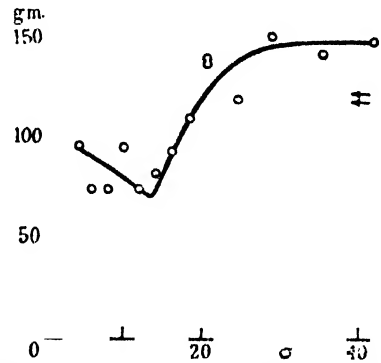


FIG. 4.—Maximum tension of the reflex response evoked by  $C_2$  (ordinates) plotted against the interval in sigma by which it follows  $C_1$  (abscissae). The tensions of the reflexes evoked by  $C_2$  alone are shown by the two arrows.

such facilitation would decrease as the  $C_1C_2$  interval lengthened, and it seems likely that the "minimum" would be due to this diminution of the facilitation before the recovery from the unresponsiveness was far advanced. In three experiments this explanation was supported by observations which showed that, as the interval between two centripetal volleys (each alone subliminal) was increased, the resulting facilitation diminished over a range of intervals at least as long as that found for the "minimum."

More convincing evidence is furnished by the following experimental procedure. When an antidromic volley was set up so as to reach the motoneurons

between  $C_1$  and  $C_2$  (the  $C_1C_2$  interval being less than that of the "minimum"), it was found that the response to  $C_2$  was almost completely abolished, even when the refractory period following the antidromic volley could have had very little effect on it (Eccles, 1931, p. 567). Now it has been shown that an antidromic volley removes all preformed central excitatory state (Eccles, 1931), hence it can be concluded that the response to  $C_2$ , when it followed  $C_1$ , must have been dependent on the central excitatory state produced by  $C_1$ , i.e., that it was due to facilitation of the motoneurons of  $C_1$ 's subliminal fringe.

The period of unresponsiveness may be due either to a refractory period or to an inhibition. In the former case the period of unresponsiveness would be limited to those motoneurons from which  $C_1$  had evoked a reflex discharge. If  $C_1$  were made weaker,  $C_2$  being left unaltered, the response to  $C_2$  should increase *pari passu* at any particular  $C_1C_2$  interval long enough to discount the complicating effects of facilitation.

In some experiments (3) this result has been obtained, but there have been two definite exceptions. Thus Plate 38, fig. 5, shows part of a series in which  $C_1$  was much weaker than  $C_2$ ; nevertheless the response to  $C_2$  was so greatly reduced that the combined response to  $C_1 + C_2$  was, at certain intervals, much less than that to  $C_2$  alone. It is clear that  $C_1$  had produced a period of unresponsiveness in many motoneurons which did not discharge reflexly; motoneurons which consequently could not have become refractory. Those motoneurons must, therefore, have been inhibited by  $C_1$ , and the inhibitory nerve fibres in the afferent nerve must have had a lower threshold than the excitatory. In the other experiment the reverse was the case. A powerful  $C_1$  prevented almost all response to  $C_2$ , while a weaker  $C_1$ , though itself still evoking a considerable reflex response, had very little effect on the response to  $C_2$  at similar  $C_1C_2$  intervals. In this case, too, the reduction of the reflex response must have been due to inhibition.

The failure to show that inhibition was the cause of the reduction of the reflex response in the other experiments may have been due to the similarity of the threshold of the excitatory and inhibitory fibres; but, until further evidence is obtained, the possibility of refractory period cannot be excluded in these cases.

In many experiments the condition of unresponsiveness described in this section eventually passed over to an unresponsiveness of long duration such as will be dealt with in the next section. In those experiments where observations were made during the period of transition this change seemed to be due, not to a gradual lengthening of the original period of unresponsiveness, but

to the gradual development of a distinct process having a long duration even at its inception. In many experiments this change occurred before it was possible to obtain any evidence on the nature of the shorter type of unresponsiveness. Changes of a similar kind during the course of experiments were observed by Forbes and co-workers (1928).

When  $C_1$  was set up in one afferent nerve and  $C_2$  in another, results have been obtained similar to those where  $C_1$  and  $C_2$  were set up in the same afferent with the single difference that the maximal facilitation at short  $C_1C_2$  intervals was observed when  $C_1$  and  $C_2$  were set up simultaneously (cf. Eccles and Sherrington, 1930, a).

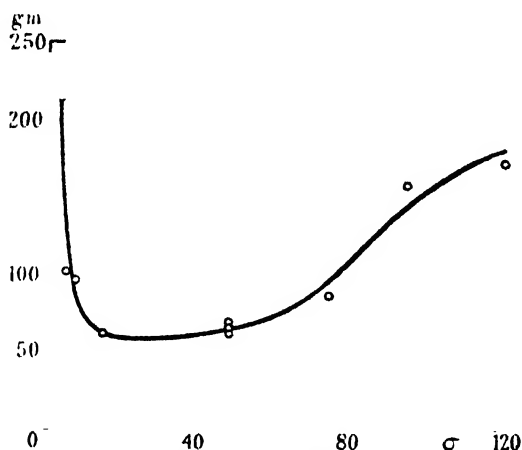
### 3. *The Reflex Responses Evoked by the Second Centripetal Volley is Diminished by the First even at Intervals longer than 80 $\sigma$ .*

Series of observations conforming to this type were the most common, being obtained in over 20 experiments (cf. Gerard and Forbes, 1928). The experimental results resemble those of the last section in exhibiting a facilitation of  $C_2$  by  $C_1$  at short  $C_1C_2$  intervals. With longer intervals there was a minimum which was, however, much more prolonged, and at still longer intervals recovery set in, but was not complete until the  $C_1C_2$  interval was at least 80  $\sigma$  and sometimes more than 400  $\sigma$ .

In almost all experiments it was quite easy to show that the unresponsiveness following  $C_1$  was not restricted to those motoneurones which had discharged reflexly. Thus in Plate 38, fig. 6,  $C_1$  was made so weak that it evoked a reflex discharge from very few motoneurones; nevertheless the much stronger reflex response evoked by  $C_2$  was greatly reduced when the  $C_1C_2$  interval was 49  $\sigma$  or 75  $\sigma$  (observations 1 and 3), and there was a considerable reduction even at intervals of 120  $\sigma$  and 96  $\sigma$  (observations 2 and 4). There can be no doubt that the response to  $C_2$  was inhibited by  $C_1$  (cf. p. 546) and that the threshold of the inhibitory fibres was lower than the excitatory.

The size of the response to  $C_2$  is plotted against the  $C_1C_2$  intervals in fig. 5. Inhibition was very slight when the  $C_1C_2$  interval was 4  $\sigma$ , and the maximum inhibition was not present until the  $C_1C_2$  had been increased to nearly 20  $\sigma$ . Recovery is observable at intervals longer than 50  $\sigma$  but was still not complete at 120  $\sigma$ . Very similar types of curves have been obtained when the inhibition has been exerted by a centripetal volley in a contralateral afferent nerve, though no inhibition was usually observable under these circumstances until the  $C_1C_2$  interval was at least 8  $\sigma$  (Samojloff and Kisseleff, 1927; Eccles and Sherrington, 1931, d).

When an antidromic volley "backfired" into the motoneurones via the nerve fibre (Eccles, 1931) is timed so that it reaches the motoneurones between



**FIG. 5.**—Maximum tension of the reflex response evoked by  $C_1 + C_2$  (ordinates) plotted against the stimulus-interval in sigmata. The tension of the reflex evoked by  $C_2$  alone is indicated by the arrow.

$C_1$  and  $C_2$ , it is found that the ipsilateral inhibition is entirely unaffected. In this respect also it resembles contralateral inhibition (Eccles and Sherrington, 1931, *d*).

Again an antidromic volley which is timed so that it just precedes  $C_1$  will greatly reduce the reflex response to  $C_1$  on account of the refractory period of the motoneurones set up by the antidromic volley. Since the reduction by  $C_1$  of the response to  $C_2$  is not thereby affected, the refractory period following the reflex discharge cannot be responsible for this reduction.

Thus it can be concluded from the experiments, that, when  $C_1$  diminishes the reflex response evoked by  $C_2$  at intervals longer than  $80\sigma$ , such diminution is due to inhibition. The threshold of the inhibitory fibres of the peripheral afferent nerve varies independently of the threshold of the excitatory fibres, hence the transitions from one type to another observed during an experiment (see p. 546).

The comparatively short-lasting inhibition described in the preceding section is probably distinct from the inhibition described in this section, for the transition from the shortlasting type (complete recovery in  $40\sigma$ ) to the long-lasting type (recovery not complete in  $120\sigma$ ) was observed in one experiment. The short-lasting type of inhibition, however, gave results similar to those

obtained with the long-lasting type in both the experiments with the antidromic volleys described above.

When  $C_1$  was set up in one afferent nerve and  $C_2$  in another, the long-lasting type of inhibition was frequently present. It was often more marked under these conditions than when  $C_1$  and  $C_2$  were both set up in the same afferent, but sometimes the reverse was the case. Also the inhibition exerted by a centripetal volley in one afferent on the motoneurons excited from another afferent often differed greatly in duration and intensity from the analogous inhibition found when the order of the centripetal volleys was reversed. In all these cases the conditions present would presumably depend on the distribution of the inhibitory fibres in the one afferent nerve to the motoneurons excited by impulses in that afferent and to the motoneurons excited from the other afferent.

The possible presence of inhibition was suggested by Eccles and Sherrington (1930, *a*) when attempting to explain the results of the interaction of two centripetal volleys each alone subliminal. In view of the present findings it seems very likely that the inhibition produced by one or both centripetal volleys would explain the asymmetry of many of the facilitation curves (*e.g.*, figs. 12, 13, 14, 15, 16, 17, and 18). In all these cases the centripetal volley which was leading on the right hand side of the figure probably inhibited the facilitation which would otherwise have occurred.

When the response to  $C_2$  was reduced by the inhibition set up by  $C_1$ , it has been found that the response to a third centripetal volley  $C_3$  was also reduced at least as much as if it were preceded by only  $C_1$  or  $C_2$ , unless the  $C_2C_3$  interval was very short ( $8\sigma$  or less). Then  $C_3$  evoked a greater response than when preceded by  $C_1$  alone. It may be concluded that  $C_2$  was effective in reducing the inhibitory effect of  $C_1$  on  $C_3$  only when it preceded  $C_3$  by such a short interval that it was able to facilitate the response to  $C_3$  without itself producing an appreciable inhibition of  $C_3$ . At longer  $C_2C_3$  intervals the inhibition produced by  $C_2$  doubtless summed with that produced by  $C_1$  in a way comparable with that found for contralateral inhibition (Eccles and Sherrington, 1931, *d*).

It was not surprising in these cases to find that, though the first centripetal volley of a repetitive series evoked a large reflex response, each subsequent volley produced either a very small response or no response at all. The effect of each volley had been reduced by the inhibition of the preceding volleys. This type of reflex response to tetanic stimulation has previously been described and has been called the "jet" form of reflex (Cooper, Denny-Brown, and Sherrington, 1927). On account of the small inhibitory effect produced by

many centripetal volleys (*cf.* p. 539) the "jet" form of response to repetitive volleys has been particularly common when the volleys were weak. With more powerful volleys a small amount of inhibition would be almost ineffective and a sustained response has been present in such cases.

#### IV. DISCUSSION.

The experimental observations leave no doubt that, when two centripetal volleys are set up in quick succession, the response evoked by the second is largely due to the discharge from the facilitated motoneurones of the subliminal fringe. The small response elicited by a stimulus applied to an afferent nerve at "the least interval for muscular summation" after a previous stimulus is due to the discharge of motoneurones which did not respond to the centripetal volley set up by the first stimulus (Forbes, Querido, Whitaker and Hurxthal, 1928). "The least interval" is that interval after the first stimulus at which a second stimulus can set up a centripetal volley sufficiently powerful to be able to increase the c.e.s. of these motoneurones to threshold. Thus the duration of "the least interval" for muscular summation in the reflex arc is more closely related to the refractory period of the afferent nerve than it is to any process in the central part of the reflex arc.

The present conceptions of reflex activity allow two possible explanations for the period of unresponsiveness which follows a centripetal volley: refractory period, and inhibition. With the former only those motoneurones which responded by a reflex discharge would be affected; with the latter there would be no such discrimination.

Using this criterion the experimental observations show that the longer periods of unresponsiveness (type 3) which followed a centripetal volley were due to inhibition, and this was confirmed by other types of experiment. Refractory period would, of course, be present in those motoneurones which responded to a centripetal volley, but it would be masked by the more prolonged inhibition. When the period of unresponsiveness was shorter (types 1 and 2), it has been difficult to distinguish between refractory period and inhibition. In two experiments inhibition has been shown to be the cause of an unresponsiveness lasting from  $40\sigma$  to  $50\sigma$ ; but no such proof has been forthcoming in other experiments described under type 2, though inhibition was not thereby excluded. An inhibition with a duration of only  $40\sigma$  to  $50\sigma$  is so much shorter than the usual forms of ipsilateral or contralateral inhibitions (shortest duration  $80\sigma$ ) that it seems to belong to a distinct type. This view was supported by the transition from the short-lasting to the long-

lasting type of inhibition which was observed to occur during the course of one experiment.

It is probable that the duration of the refractory period of the reflex arc would not vary greatly from experiment to experiment, for in peripheral nerve the range of variation is small. Now if all the observations of type 3 and those two of type 2 which are known to be due to inhibition are excluded, there was only one series of observations (duration  $50\sigma$ ) where the duration of the period of unresponsiveness was greater than  $30\sigma$ . It seems likely that inhibition was also present in this case, but unfortunately the transition to type 3 took place before this could be investigated.

Thus it is possible to conclude that the refractory period of the reflex arc has a total duration of not more than  $30\sigma$ . Inhibition has, of course, not been excluded as an explanation of the unresponsiveness in all the cases of type 2; but in the cases of type 1 it seems likely that inhibition plays no prominent part. Especially is this so in those three experiments which displayed the marked facilitation. In the other three experiments of type 1 (where facilitation was not marked) observations showed that the period of unresponsiveness was not longer than  $15.8\sigma$ . At intervals shorter than this facilitation was an interfering factor. *Thus out of the results of the whole experimental series, it seems likely that  $15.8\sigma$  is the most significant superior limit which can be placed to the duration of the relatively refractory period of the reflex arc.*

The subject has, however, also been approached from a different aspect in another series of experiments which are described in the next paper (Eccles, 1931). When an antidromic impulse passes into a motoneurone via the motor nerve fibre, it is there shown that a period of unresponsiveness to normal reflex stimuli is set up in the motoneurone. From experimental evidence it is induced that this unresponsiveness is a refractory period—at first absolute, later relative. Further, other experiments showed that such an antidromic impulse travelled as far as to affect the locus of formation of c.e.s. which is probably the synapse. Now, on general grounds, it may be assumed that a nerve impulse passing over any part of a motoneurone or its dendrites sets up a refractory period which is *independent* of the direction of its travel. Hence, so far as the motoneurone is concerned, *it may be taken that the total duration of the relatively refractory period following a normal reflex discharge is identical with that following an antidromic volley, namely about  $10.5\sigma$  (Eccles, 1931, p. 567).*

In the representation of the central path of a reflex arc in fig. 6 no internuncial neurones are shown because no evidence of their presence could be obtained



when the centripetal volleys were not large (Eccles, 1931, p. 576). There rise to mind three possible factors which could control the duration of the relatively

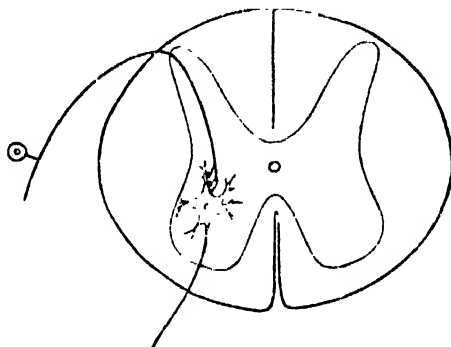


FIG. 6.—Diagram showing a simple reflex arc in the spinal cord.

refractory period of this reflex arc: the relatively refractory period of the afferent central terminal, the temporary partial "exhaustion" of the mechanism for producing c.e.s., and the relatively refractory period of that part of the motoneurone which is excited by the c.e.s. If c.e.s. is due to changes in the interfacial membranes of the synapse (Eccles and Sherrington, 1931, c) it does not seem likely that c.e.s. production can vary independently of the first and third factors which depend, respectively, on the afferent and efferent sides of this membrane.\* Now it has just been inferred that the third factor is about  $10\cdot5\sigma$  in duration. The value of the first factor can only be surmised, but, in view of the optimum interval for facilitation between two subliminal centripetal volleys ( $3\sigma$  to  $6\sigma$ ), it is unlikely to be significantly longer than the third factor (*cf.* Bremer, 1930, b). In this connection it is interesting to note that Gasser and Erlanger (1930) have been unable to detect any difference between the absolutely refractory periods of all the fibres of the A group, and also between the relatively refractory periods of the  $\alpha$  and some of the  $\beta$  group. It seems that the refractory periods of nerve fibres vary to a much less

\* The following experiment also shows that any partial "exhaustion" of the mechanism for producing c.e.s. has no significant duration. When a just subliminal centripetal volley was followed by a slightly stronger centripetal volley in the same afferent nerve at intervals longer than those at which there was facilitation, there was no reduction of the weak reflex response to the second volley in those cases where inhibition was not present. In this way it was shown that the capacity of a weak centripetal volley to produce c.e.s. was unaffected by a preceding just-subliminal volley at intervals from  $10\sigma$  to beyond  $100\sigma$ . i.e., there was no appreciable "exhaustion" at these intervals.

degree than the thresholds of excitation or the velocities of propagation of impulses.

*It may be provisionally concluded that the relatively refractory period of the reflex arc of the ipsilateral flexor reflex is likely to approximate to  $10.5\sigma$ . The observations which gave an upper limit of  $15.8\sigma$  would not be in disagreement with this value, though the periods of unresponsiveness of those observations of type 2 which lasted as long as  $30\sigma$  receive no satisfactory explanation. Further investigation is necessary in order to throw light on the nature of these cases.*

Though it has been found that motoneurones respond to each of two centripetal volleys only  $2.8\sigma$  apart, it has been pointed out that there is almost certainly a longer interval between the successive responses of the motoneurones, and so these observations do not give reliable evidence on the duration of the absolutely refractory period of the reflex arc. Arguments similar to the above (see p. 552) make it likely that the absolutely refractory period would not be due to the c.e.s. mechanism independently of the refractory periods of the afferent and efferent parts of the synaptic membrane. The upper limit to the latter has been found to be as short as  $2.4\sigma$  (Eccles, 1931, p. 568), and the absolutely refractory period of the former cannot be appreciably longer than this on account of the shortness of "the least interval for muscular summation" (see p. 535); the brevity of the least interval is, it is true, due to the response of motoneurones of the subliminal fringe, but the second centripetal volley must be able to reach the synapses in order to produce the c.e.s. necessary for facilitation. The interval between the arrival of the successive centripetal impulses at any synapse will be longer than the stimulus interval on account of the delay of an impulse travelling in the relatively refractory period, but, for the distances travelled, this delay is not likely to be more than  $1\sigma$  to  $2\sigma$  (Gasser and Erlanger, 1925). Thus a centripetal impulse set up in an afferent nerve fibre  $1\sigma$  after a previous centripetal impulse would reach a synapse only  $2\sigma$  to  $3\sigma$  later than that impulse. The absolutely refractory period of the afferent pathway would in no place be shorter than this interval. It may be concluded that no part of the reflex arc is likely to have an absolutely refractory period longer than  $2\sigma$  to  $3\sigma$ .

It does not follow from this, however, that a reflex discharge from a motoneurone can be set up  $2\sigma$  to  $3\sigma$  after a previous discharge. From certain experimental results it has been induced (Eccles and Sherrington, 1931, b) that in any motoneurone there is a removal of preformed c.e.s. by a reflex discharge, and that c.e.s. must be built up to threshold before a second

reflex discharge can be set up. The shortest time necessary for this must be added on to the duration of the absolutely refractory period in order to arrive at the least interval between two consecutive reflex discharges of a motoneurone. Thus  $3.9\sigma$  was the shortest interval after an antidromic volley at which a reflex discharge could be set up (Eccles, 1931, p. 568). Such a short interval was only obtained by the powerful excitation of the motoneurones produced by two centripetal volleys in quick succession. The comparatively weak excitation produced by a second centripetal volley would take longer to set up a reflex discharge (Eccles and Sherrington, 1931, *a*, p. 517), so it is probable that the least interval between the successive reflex discharges which are set up by two centripetal volleys would be longer than  $3.9\sigma$ .

From certain observations on the interaction between two rhythmic series of centripetal volleys in separate afferent nerves, Cooper and Denny-Brown (1929) have inferred that the impulses of one series can evoke a reflex discharge from motoneurones even when incident at less than  $1\sigma$  after impulses of the other series which likewise evoked a discharge from those motoneurones. The centripetal impulses of a single volley, however, show a considerable dispersion in their times of incidence on a motoneurone (Eccles and Sherrington, 1931, *a* ; 1931, *b* ; 1931, *c* ; Eccles, 1931) ; hence many impulses of the second volley would probably arrive at a considerable time after a reflex discharge had been set up by the preceding volley, even when the interval between the setting up of two volleys was less than  $1\sigma$ . The results, therefore, need not be interpreted as indicating that impulses incident on a motoneurone during its absolutely refractory period give rise to an excitatory state which persists until the recovery of the motoneurone allows a discharge to be set up. On analogy with observations on peripheral nerve and muscle (Lucas, 1911), however, it is possible that c.e.s. can be produced by excitatory impulses incident during the latter part of the absolutely refractory period.

#### SUMMARY.

When the interval between two centripetal volleys is short, the reflex contraction evoked by the second volley is due largely to the discharge of motoneurones which fail to respond to either volley alone (*cf.* Forbes and co-workers, 1928). The response of these motoneurones is due to summation of the subliminal excitatory effects of each volley. The least interval at which the second of two successive centripetal volleys evokes a response does not therefore serve to measure the refractory period of the reflex arc, since the second response is not from the same motoneurones as the first.

In addition to this facilitation at short intervals, a centripetal volley gives to a period of unresponsiveness of motoneurons as shown by the smallness of the reflex response evoked by a later centripetal volley. Three types of unresponsiveness have been met with:—

1. Recovery complete in less than  $16\sigma$ . Here the unresponsiveness is almost certainly a true refractory period following the reflex discharge set up by the first volley. Its duration is in agreement with the average value of  $10.5\sigma$  obtained for the duration of the refractory period of motoneurons following an antidromic volley.
2. Recovery complete in less than  $50\sigma$ . In the more prolonged cases the unresponsiveness is due to an inhibition set up by some of the impulses of the first volley. When recovery is complete in less than  $30\sigma$ , it is not known whether the unresponsiveness is due to inhibition or refractory period.
3. Recovery not complete for more than  $80\sigma$ . This prolonged unresponsiveness is due to the inhibition produced by some of the impulses in the first volley. This inhibition is similar to the inhibition produced by stimulation of contralateral nerves.

From theoretical considerations it is deduced that the duration of the relatively refractory period following an antidromic volley ( $10.5\sigma$ ) is likely to be identical with the duration of the relatively refractory period of the reflex arc.

One of us (J.C.E.) wishes to express his gratitude to the Medical Research Council for a personal grant, and we have to thank the Royal Society and the Rockefeller Foundation for grants towards the apparatus used in this research.

#### REFERENCES.

- Adrian, E. D., and Forbes, A. (1922). 'J. Physiol.,' vol. 56, p. 301.  
 Adrian, E. D., and Olmsted, J. M. D. (1922). 'J. Physiol.,' vol. 56, p. 426.  
 Bremer, F. (1930, a). 'C. R. Soc. Biol.,' vol. 103, p. 509.  
 Bremer, F. (1930, b). 'C. R. Soc. Biol.,' vol. 103, p. 513.  
 Cooper, S., and Denny-Brown, D. E. (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 363.  
 Cooper, S., Denny-Brown, D. E., and Sherrington, C. S. (1927). 'Proc. Roy. Soc.,' B, vol. 101, p. 262.  
 Denny-Brown, D. E., and Sherrington, C. S. (1928). 'J. Physiol.,' vol. 66, p. 175.  
 Eccles, J. C. (1931). 'Proc. Roy. Soc.,' B, vol. 107, p. 557.  
 Eccles, J. C., and Hoff, H. E. (1931). *To be published shortly.*  
 Eccles, J. C., and Sherrington, C. S. (1930, a). 'J. Physiol.,' vol. 69, p. 1.  
 Eccles, J. C., and Sherrington, C. S. (1930, b). 'Proc. Roy. Soc.,' B, vol. 106, p. 326.

- Eccles, J. C., and Sherrington, C. S. (1930, c). 'Proc. Physiol. Soc.,' p. xxv; 'J. Physiol.,' vol. 70.
- Eccles, J. C., and Sherrington, C. S. (1931, a). 'Proc. Roy. Soc.,' B, vol. 107, p. 511.
- Eccles, J. C., and Sherrington, C. S. (1931, b). 'Proc. Roy. Soc.,' B, vol. 107, p. 586.
- Eccles, J. C., and Sherrington, C. S. (1931, c). 'Proc. Roy. Soc.,' B, vol. 107, p. 597.
- Eccles, J. C., and Sherrington, C. S. (1931, d). *In the course of publication.*
- Forbes, A. (1922). 'Phys. Rev.,' vol. 2, p. 361.
- Forbes, A., and Gregg, A. (1915). 'Amer. J. Physiol.,' vol. 37, p. 118.
- Forbes, A., Querido, A., Whitaker, L. R., and Hurxthal, L. M. (1928). 'Amer. J. Physiol.,' vol. 85, p. 432.
- Gasser, H. S., and Erlanger, J. (1925). 'Amer. J. Physiol.,' vol. 73, p. 613.
- Gasser, H. S., and Erlanger, J. (1930). 'Amer. J. Physiol.,' vol. 94, p. 247.
- Gerard, R. W., and Forbes, A. (1928). 'Amer. J. Physiol.,' vol. 86, p. 186.
- Lucas, K. (1911). 'J. Physiol.,' vol. 43, p. 46.
- Samojloff, A., and Kisseleff, M. (1927). 'Pflügers Arch.,' vol. 215, p. 699.
- Sherrington, C. S. (1921). 'Proc. Roy. Soc.,' B, vol. 92, p. 245.
- Sherrington, C. S. (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 332.
- Sherrington, C. S., and Sowton, S. C. M. (1915). 'J. Physiol.,' vol. 49, p. 331.

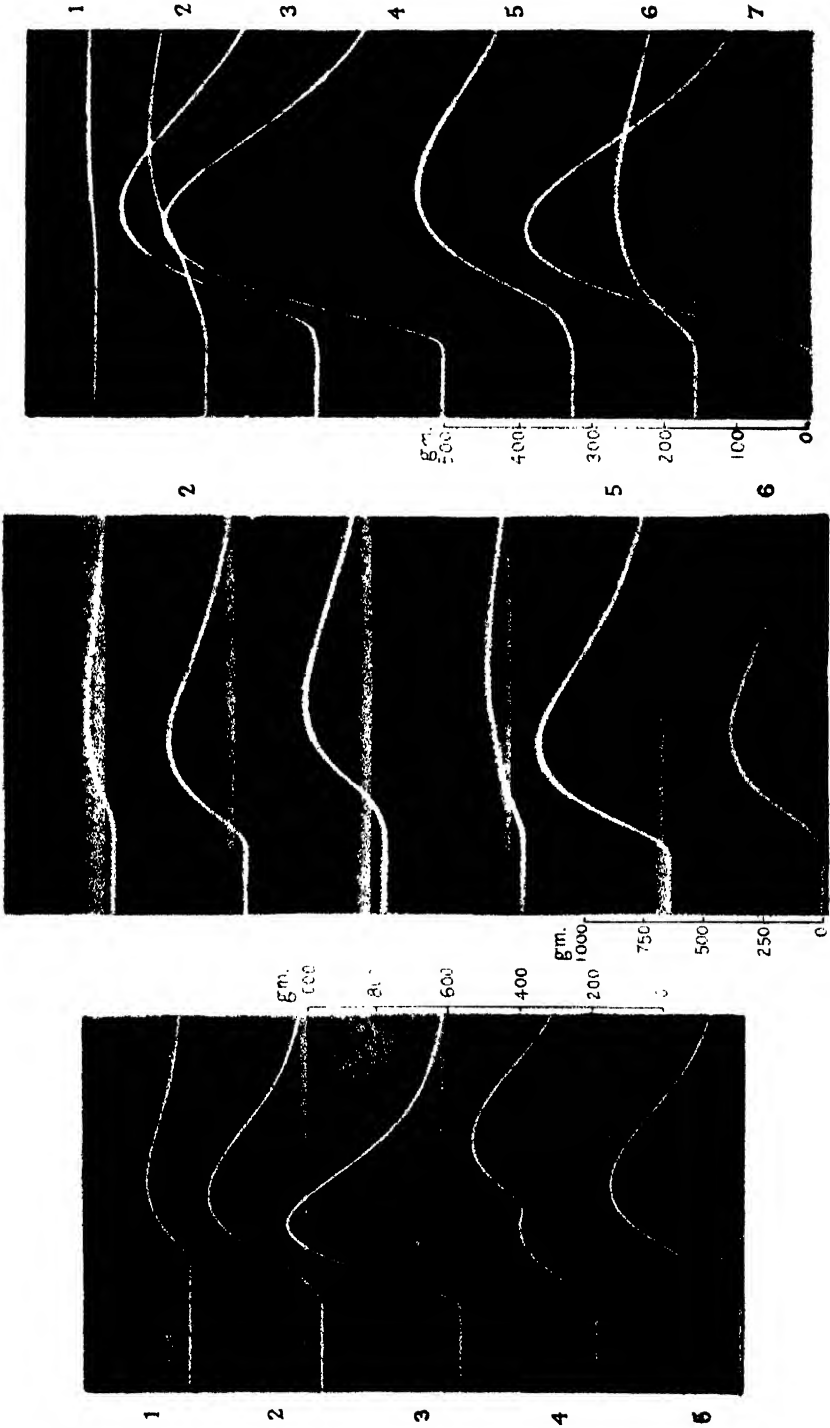
## EXPLANATION OF PLATES.

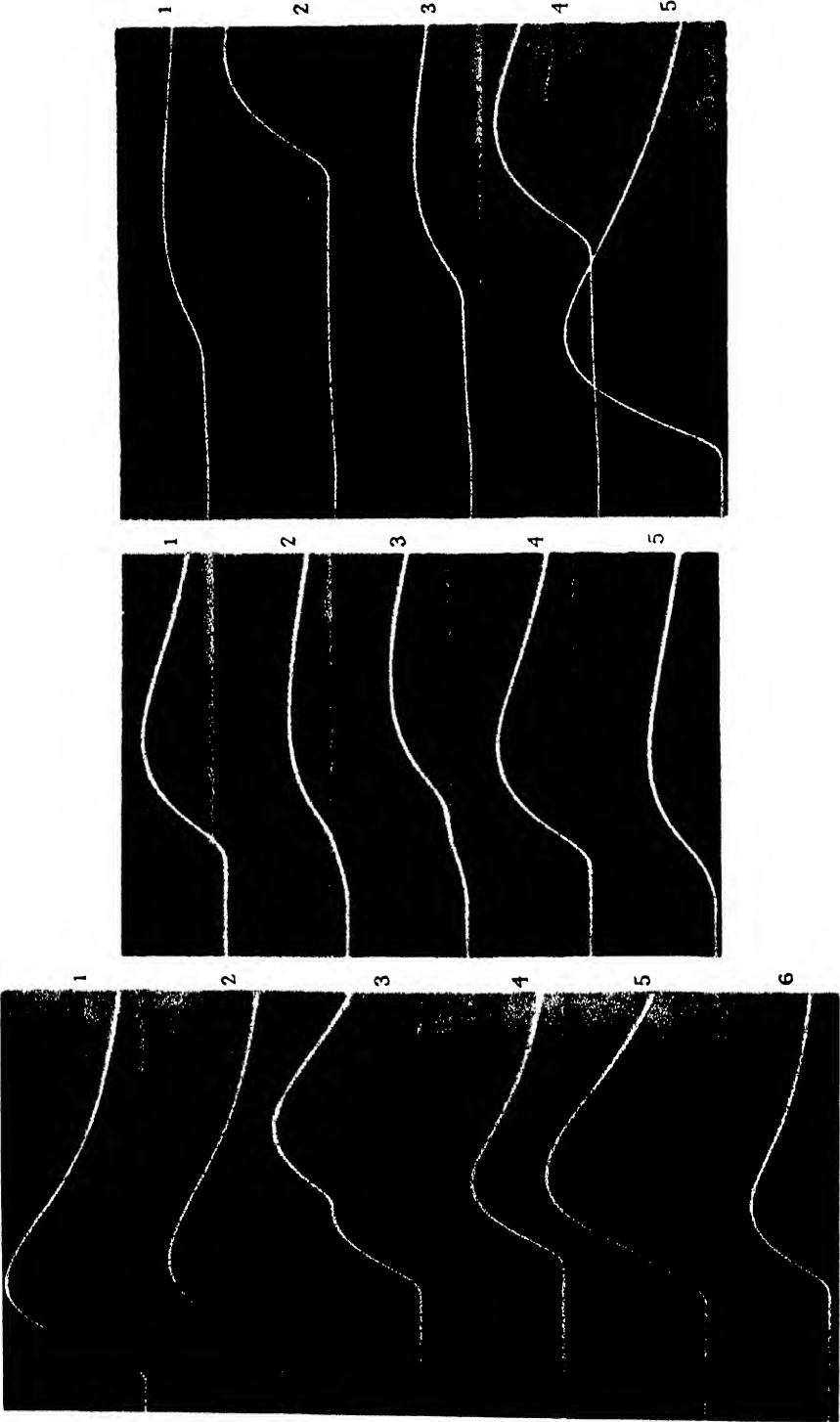
## PLATE 37.

- FIG. 1.—Reflex responses of tibialis anticus muscle evoked by one or two centripetal volleys in popliteal nerve as follows:—1,  $C_1$  alone. 2,  $C_1$  15·8  $\sigma$   $C_2$ . 3,  $C_1$  5·9  $\sigma$   $C_2$ . 4,  $C_1$  40·8  $\sigma$   $C_2$ . 5,  $C_1$  19·8  $\sigma$   $C_2$ . The white lines of the myographic record commence below the corresponding electrical record (dark lines). Time. 1 d.v. = 10  $\sigma$ . Tension scale at side.
- FIG. 2.—As in fig. 1 in another experiment. The intervals are as follows:—1,  $C_2$  alone. 2,  $C_1$  4  $\sigma$   $C_2$ . 3,  $C_1$  19·8  $\sigma$   $C_2$ . 4,  $C_1$  40·8  $\sigma$   $C_2$ . 5,  $C_1$  7·9  $\sigma$   $C_2$ . 6,  $C_1$  15·8  $\sigma$   $C_2$ . Tension scale at side.
- FIG. 3.—As in figs. 1 and 2 in another experiment. The intervals are as follows:—1,  $C_2$  alone. 2,  $C_1$  32·2  $\sigma$   $C_2$ . 3,  $C_1$  13·8  $\sigma$   $C_2$ . 4,  $C_1$  7·9  $\sigma$   $C_2$ . 5,  $C_1$  19·8  $\sigma$   $C_2$ . 6,  $C_1$  40·8  $\sigma$   $C_2$ . 7,  $C_1$  5·9  $\sigma$   $C_2$ . Tension scale at side.

## PLATE 38.

- FIG. 4.—As in figs. 1 and 3 (Plate 37) in another experiment. The intervals are as follows:—1,  $C_1$  9·9  $\sigma$   $C_2$ . 2,  $C_1$  13·8  $\sigma$   $C_2$ . 3,  $C_1$  41  $\sigma$   $C_2$ . 4,  $C_1$  alone. 5,  $C_1$  19·8  $\sigma$   $C_2$ . 6,  $C_2$  alone. Tension scale as for Plate 37, fig. 3.
- FIG. 5.—Reflex responses of tibialis anticus muscle evoked by one or two centripetal volleys in popliteal nerve, the first volley being much smaller than the second. The intervals are as follows:—1,  $C_1$  alone. 2,  $C_1$  19·8  $\sigma$   $C_2$ . 3,  $C_1$  32·2  $\sigma$   $C_2$ . 4,  $C_2$  alone. 5,  $C_1$  11·8  $\sigma$   $C_2$ . Tension scale as for Plate 37, fig. 3.
- FIG. 6.—As in fig. 5 in another experiment. The intervals are as follows:—1,  $C_1$  49  $\sigma$   $C_2$ . 2,  $C_1$  120  $\sigma$   $C_2$ . 3,  $C_1$  75  $\sigma$   $C_2$ . 4,  $C_1$  96  $\sigma$   $C_2$ . 5,  $C_1$  alone. Tension scale as for Plate 37, fig. 3.





*Studies on the Flexor Reflex.—III. The Central Effects Produced by an Antidromic Volley.*

By J. C. ECCLES, M.A., D.Phil., Research Fellow of Exeter College,  
Physiological Laboratory, Oxford.

(Communicated by Sir Charles Sherrington, F.R.S.—Received January 2, 1931.)

[PLATES 39, 40.]

CONTENTS.

	PAGE
I. Introduction .....	557
II. Methods .....	559
III. Results .....	559
1. The effect of an antidromic volley on the reflex response (reflex twitch) evoked by a single centripetal volley .....	560
i. The reduction of the reflex response .....	561
ii. The lengthening of the latent period .....	565
iii. Discussion.....	566
iv. Conclusions .....	570
2. The effect of an antidromic volley on the central excitatory state .....	570
i. Introduction .....	570
ii. Effect on the facilitation between two subliminal centripetal volleys.....	571
iii. Effect on the facilitation between the subliminal fringes of two centripetal volleys.....	572
iv. Conclusions .....	575
3. The time-factor in the formation of c.e.s. ....	576
i. Experimental observations.....	576
ii. Discussion.....	579
iii. Conclusions .....	580
4. The time-factor in the action of an antidromic volley on the central effect of a centripetal volley .....	581
i. Discussion.....	581
ii. Conclusion.....	583

I. INTRODUCTION.

In 1822 Magendie demonstrated that no reflex activities were evoked by impulses passing into the spinal cord by a ventral root. This result has been



confirmed and extended by other investigators who showed that no action-currents can be detected in other nerves when the central end of a cut ventral root is stimulated (Mislowski, 1895 ; Bernstein, 1898).

Attempts have been made to account for the irreversibility of conduction in the reflex arc by postulating a "dynamical polarisation" of the nerve cell so that the conduction would be solely from dendrite to axon, never the reverse (Cajal, 1891 ; van Gehuchten, 1900). The antidromic impulses "back-fired" into a motoneurone might, however, be blocked at the synapse (Sherrington, 1900, p. 798). It seems unlikely that the conduction of nerve impulses in the cell body and dendrites of a motoneurone would differ fundamentally from the conduction in peripheral nerve fibres, *e.g.*, that impulses passing along the dendrites would suffer an irreciprocal decrement. In the present paper it has been assumed that antidromic impulses in motor nerve fibres are blocked at the synapses of the motoneurones.

Denny-Brown (1928, 1929) has investigated the effect produced in motoneurones by antidromic impulses in the ventral root fibres. When an antidromic volley was "backfired" into the extensor centre during a crossed extensor reflex,\* there was found to be a cessation of reflex discharge for about 40  $\sigma$ . At first this blocking of reflex discharge was considered to be due to a central refractory state set up by the antidromic volley (1928, p. 328). Later he suggested that there was a momentary exhaustion of the accumulated exciting agent (c.e.s.) of the motoneurones (1929, p. 274).

Certain results obtained from stimulating the intact median nerve in human beings were thought by Hoffmann and Keller (1928) to be the result of a type of refractory period set up by antidromic impulses in the motoneurones. Centripetal impulses in afferent nerve fibres would, however, also be incident on the motoneurones, and their effect was not excluded.

Experiments have been recorded, *e.g.*, Gerard and Forbes (1928), in which the intact motor nerve of a flexor muscle was stimulated by a single break-shock. The antidromic volley in the motor nerve fibres did not block the reflex response evoked by the centripetal volley in the afferent nerve fibres. This shows that any block set up by the antidromic volley had a relatively short duration.

The following investigation is an attempt to elucidate the effect which an antidromic volley has on the motoneurones of a flexor muscle (tibialis anticus).

\* A single maximal break-shock was applied to the intact motor nerve, and the centripetal impulses set up in the afferent nerve fibres were blocked by previous section of the appropriate dorsal roots.

## II. METHOD.

The general experimental technique has already been described in detail (Eccles and Sherrington, 1931, *a*). Reflex responses have been evoked in tibialis anticus muscle by break-shocks applied to either popliteal or posterior tibial nerves, except in the case of experiment 30.7.30, where internal saphenous nerve was stimulated. The motor electrodes have been placed on the peroneal nerve after all peripheral branches except those to tibialis anticus had been cut. All the afferent nerve fibres of peroneal nerve have been cut centrally and this has been confirmed in every experiment by *post-mortem* dissection. Usually the 6th and 7th post-thoracic dorsal roots have been cut extradurally and the branch which the peroneal nerve may receive from the 8th post-thoracic spinal nerve has been cut if present. In some experiments the 6th post-thoracic dorsal root only has been cut, and the branches which the peroneal nerve received from both the 7th and 8th post-thoracic nerves were then severed in the sacral plexus. All the nerves of the limb except peroneal were divided in all experiments, so the muscular contraction resulting from stimulation of the intact motor nerve would set up no centripetal impulses which could reach the spinal cord.

No attempt has been made to cut centrally the sympathetic fibres of the peroneal nerve. But in all experiments the break-shocks (coreless coil) applied to the intact motor nerve were just supramaximal for the motor nerve fibres, and consequently much too weak to stimulate any sympathetic nerve fibres.

It can be concluded therefore, that, when the intact motor nerve was stimulated, the only impulses to reach the spinal cord would be the antidromic impulses in the motor nerve fibres. In those experiments where the branch from 7th post-thoracic spinal nerve to peroneal was cut, some of the motor nerve fibres of tibialis anticus would be severed. The corresponding motoneurons would not be reached by an antidromic volley, but this would be immaterial because their reflex responses would also be blocked.

## III. RESULTS.

When a single maximal (for the motor fibres) break-shock is applied to the intact motor nerve by electrodes *a* (fig. 1), it sets up two volleys of impulses which travel in opposite directions. One, the centrifugal volley, passes to the muscle and excites it to respond by a maximal twitch; the other, the centripetal, passes to the spinal cord. Since all the afferent nerve fibres have been cut, the only centripetal impulses to reach the spinal cord will be those in the

motor nerve fibres; henceforth such impulses will be called antidromic\* impulses, because their direction of travel is the reverse from normal.

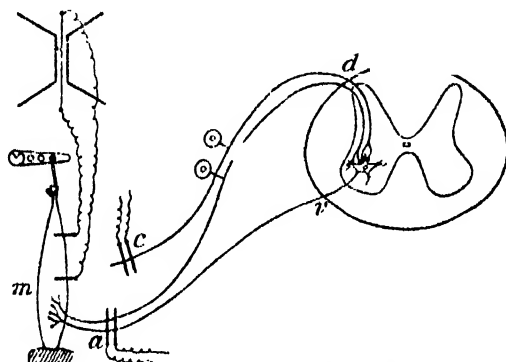


FIG. 1.—Diagram of reflex pathway and recording systems.

A maximal motor twitch has invariably been the muscular response evoked by a single maximal break-shock applied through electrodes *a*, i.e., *there has never been any evidence that an antidromic volley evoked a discharge of nerve impulses from the motoneurons which it reached*. Therefore in order to obtain positive evidence of the effect of antidromic impulses on motoneurons it is necessary to investigate the behaviour of these motoneurons to centripetal impulses (in afferent nerve fibres) at various times after the arrival of the antidromic impulses.

In the simplest type of such experiments a single antidromic volley is followed at various intervals by a single centripetal volley which is set up by a break-shock applied by electrodes *c* (fig. 1) to a suitable afferent nerve. For convenience the centripetal volley will from now on be called C, or, if two centripetal volleys are referred to,  $C_1$  and  $C_2$ , and the antidromic volley will be called A.

### 1. *The Effect of an Antidromic Volley on the Reflex Response (Reflex Twitch)† Evoked by a Single Centripetal Volley.*

If C reaches the motoneurons at a sufficiently long interval after A, it produces a reflex response which is unaffected by the precedence of A. As the

\* I am indebted to Professor Sherrington for suggesting the word "antidromic." The word "antidrome" was applied by him (1897) to the conduction of impulses down the tracts of Goll and Burdach when these were stimulated in the cervical region. The vaso-dilator impulses in dorsal root fibres were later (1901) called "antidromic" by Bayliss, at the suggestion of Langley.

† The effect of the antidromic volley on reflex responses in which there is more than one reflex discharge from many motoneurons (after-discharge) will be considered in another paper (Eccles and Sherrington, 1931, c).

AC interval is reduced, the reflex becomes smaller and its latent period lengthens, until finally A prevents all response to C if such response is a reflex twitch (*vide* Eccles and Sherrington, 1931, *a*, p. 522). Part of such a series is shown in Plate 39, fig. 1. The two effects which A produces on the reflex response evoked by C will be considered in detail separately.

(i) *The Reduction of the Reflex Response*.—The action-current is not a reliable measure of the size of a reflex response, because the magnitude of the deflection depends on the temporal dispersion of the response (Forbes and Gregg, 1915). Moreover the reflex response is always superposed on the maximal motor twitch (a necessary consequence of the setting up of the antidromic volley), so its tension cannot be directly measured. It has, however, been found that such a superposed muscular contraction can be approximately calculated from the maximum tension added to the maximal motor twitch (the method of maximum tension differences\* (Eccles and Hoff, 1931)).

In fig. 2A the maximum increments of tension given by a series of reflex responses (some of which are shown in Plate 39, fig. 1) are plotted as ordinates

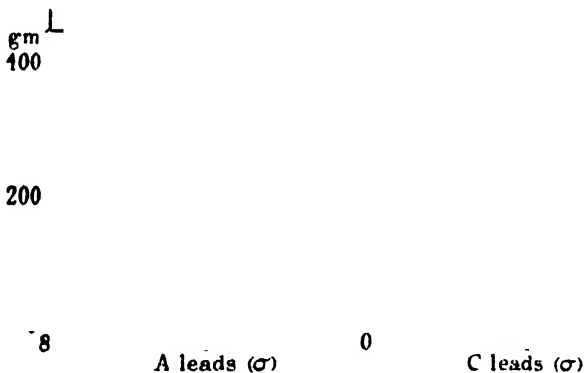


FIG. 2A.—Tensions of reflex responses (ordinates) are plotted against the respective intervals between the stimuli to the afferent and motor nerves (abscissae). The tensions of reflex responses alone are shown near the tension scale.

against the respective intervals between the setting up of A and C (abscissae). The maximum tension thus calculated is 1.27 times that developed by the

\* The maximum increment of tension given to a maximal motor twitch by a given sub-maximal twitch bears a ratio to the tension of the latter which is approximately constant for a particular muscle when the interval between the two responses is not more than 30  $\sigma$ . The value of the ratio shows a considerable amount of variation from one preparation to another. It is usually about 1.3 to 1.4, but it may be as high as 1.7 or as low as 1.1 (Eccles and Hoff, 1931).

reflex alone (individual values marked by the arrows), and, since no regular diminution is observable in fig. 2A until C was set up less than  $4\sigma$  after A, it seems most probable that the reflex was not reduced in size when A was set up  $4\sigma$  or more before C. Even when A was set up  $4\sigma$  after C, the reflex was only reduced by about 50 per cent. Fig. 2B is a more complete series from

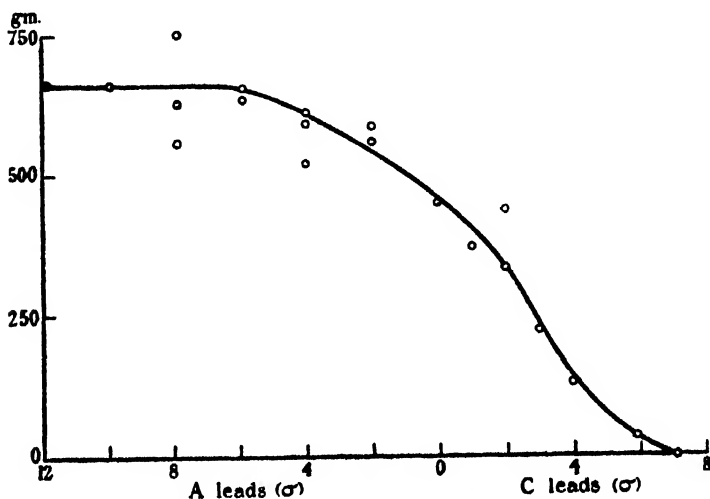


FIG. 2B.—As in fig. 2A in another experiment.

another experiment which showed no significant diminution of the reflex response until the AC interval was less than  $6\sigma$ .

The temporal relationship between events in the spinal cord is best discussed in connection with a diagram (fig. 3 shows the conditions in the experiment represented in fig. 2A) similar in construction to those already used (Eccles and Sherrington, 1931, *a* and *b*). Ordinates represent distances drawn to scale, and abscissæ time in sigmata. *cd* is the afferent nerve from the electrodes *c* to the spinal cord, and *va* is the efferent nerve from the spinal cord to the electrodes *a* (see fig. 1). The efferent nerve from *a* to the muscle is approximately represented by *am*. In those experiments where the velocities of propagation of the antidromic and centripetal volleys were not measured they were assumed to be 80 metres per second and 35 metres per second respectively (Eccles and Sherrington, 1931, *a*). The centripetal volley C is shown by  $c_1d_1$ , and A is represented by  $a_1v_1$ , or  $a_2v_2$ , or  $a_3v_3$  according to its temporal relationship to C.

When A was set up  $4\sigma$  before C, it entered the spinal cord at  $v_1$ ,  $12.6\sigma$  before the earliest reflex discharge  $v_4a_4$  passed out. This was the shortest interval, measured at *v*, between A and the passing out of a reflex discharge

**FIG. 3.**

When A was set up 4  $\sigma$  after C, it is shown by  $a_3v_3$ . The reflex discharge  $v_3a_3$  evoked by C had then a longer latent period. The shortest interval, measured at  $v$ , which was observable between A and the passing out of a reflex discharge is, therefore, shown by  $v_3v_5 = 5.0 \sigma$ . When A was set up later than  $a_3$ , it did not block all the reflex response to C (see fig. 2A), but there was no definite action-current for the small reflex discharge which occurred under such circumstances (Plate 39, fig. 1, observations 3 and 5), so the reflex latent

period could not be measured. The reflex discharge still unblocked when A was set up  $8\sigma$  after C (fig. 2A) was probably after-discharge (Eccles and Sherrington, 1931, c). The observed shortest interval,  $5\sigma$ , between the passing in of A and the passing out of a reflex discharge was thus an upper limiting value to the actual shortest interval. In all the reliable experiments which we have performed this upper limit has been between  $5.0\sigma$  and  $6.1\sigma$  (column 2, Table I).

Table I.

1	2*	3*	4*	5	6	7
Date of experiment.	Shortest interval after A at which a reflex discharge can be detected $= v_3v_5$ , fig. 3.	Shortest interval after A at which a reflex discharge is unaltered $= v_1v_4$ , fig. 3.	Longest interval after A at which a reflex discharge is reduced $= v_2v_4$ , fig. 3.	Greatest amount of lengthening of "modal" latent period.	Temporal dispersion measured as described by Eccles and Sherrington (1931, a).	"Modal" latent period of reflex.
	$\sigma$	$\sigma$	$\sigma$	$\sigma$	$\sigma$	$\sigma$
5.5.30	5.9	11.7	10.1	2.3	0.4	11.2
16.5.30	6.1	12.6	8.8	1.7	2.0	12.8
21.5.30	5.0	12.6	10.6	0.4	0.0	13.1
26.5.30†	5.0	8.0	7.2	1.8	—	8.2
	5.4	11.9	9.2	2.6	2.6	11.6
19.6.30	5.6	11.3	9.2	2.2	1.4	11.8
14.7.30	5.8	9.3	7.9	4.5	0.2	9.5
	—	11.3	—	—	—	—
17.7.30	5.3	not determined	—	1.0	3.0	13.1
28.7.30	6.0	12.5	10.8	1.6	2.7	13.5
17.10.30	5.9	11.8	9.8	0.7	1.4	12.9
27.10.30	5.0	11.6	9.6	—	1.3	10.8

\* The intervals in columns 2, 3 and 4 are calculated for the point of exit of the ventral root from the spinal cord, i.e., point *v*, fig. 3.

† The action-currents of the reflex had a double summit in this experiment. As "backfiring" showed that there was a repetitive discharge in very few motor units, the double action-current must have been due to the discharging motoneurons falling into an early and a delayed group. In the table the values for the former are given above the latter.

The time, measured at *v*, necessary for complete recovery from the period of diminished reflex response following an antidromic volley will lie between the values of columns 3 and 4, Table I, which form its respective superior and inferior limits.

It will be seen that a value of  $10.5\sigma$  is usually a fair approximation. At about this interval recovery is so perfect that even the weakest reflexes do not suffer any diminution. The lower value in column 3, experiment 14.7.30, was obtained under these conditions.

The proportion of the reflex which was unblocked at any particular AC

interval was the greater the larger the centripetal volley. This happened even when the increase in the volley was not sufficient to evoke a reflex accompanied by after-discharge. There are two possible explanations: either the additionally excited motoneurones were less susceptible to the effects of the antidromic volley than those originally excited, or the larger centripetal volley made the originally excited motoneurones more resistant to the effects of the antidromic volley. On general grounds it seems likely that the antidromic volley should raise the neurone-threshold of all the motoneurones in a similar way, so, if the additional motoneurones were less susceptible, it would have to be due to their more intense excitation. But it is most probable that the intensity of their excitation by the centripetal volley was less than that of those originally excited, for it was not raised to neurone-threshold by the smaller centripetal volley; hence the second of the two explanations is much the more likely. The larger centripetal volley increased the excitation of many motoneurones which were previously prevented from discharging by the raised neurone-threshold following the antidromic volley, with the result that the antidromic volley was now no longer able to prevent their reflex discharge.

Thus the partial reduction of a reflex by an antidromic volley is not due to an absolute blocking of the reflex discharge from some motoneurones. Motoneurones are prevented from discharging reflexly because their raised neurone-threshold is higher than the amount of excitation produced by the centripetal volley. Since some motoneurones are excited more strongly than others, some will be prevented from discharging only when the neurone-threshold is high, others even when it is raised by a comparatively small amount. In those motoneurones in which the c.e.s. is normally just sufficient to reach neurone-threshold, the smallest elevation of neurone-threshold will suffice to prevent a reflex discharge. Thus the longest AC interval at which a diminution of the reflex discharge can be detected (column 4, Table I) is a lower limit to the duration of the raised neurone-threshold following an antidromic volley. Similarly the values of column 3, Table I, give an upper limit to this duration. It can be concluded that a curve showing the reduction of the reflex response at various AC intervals, e.g., figs. 2A and 2B, is closely related to a curve showing the neurone-threshold of the motoneurones at various intervals after an antidromic volley.

(ii) *The Lengthening of the Latent Period.*—When the reflex response is considerably reduced by a preceding antidromic volley, there is also found to be a small lengthening of its latent period. This is measured (*cf.* Eccles and Sherrington, 1931, *a*) by the summits of the action-currents, because the action-



current of the immediately preceding maximal motor twitch prevents any accurate determination of the beginning of the reflex action-current. This lengthening is the more marked the shorter the interval by which the antidromic volley precedes the reflex discharge. Only in experiment 14.7.30 was the maximum alteration greater than  $3.0\sigma$  (column 5, Table I). The explanation of the lengthening will be considered later.

(iii) *Discussion*.—From histological considerations it seems likely that an antidromic impulse passing into the spinal cord through a ventral root fibre would proceed to traverse the surface of the associated anterior horn cell and reach the terminals of the dendrites of that cell.\* The limit to the passage of the antidromic impulse is presumably set by the numerous synapses bordering on the perikaryon and dendrites of the motoneurone. Beyond these synapses the antidromic impulse does not go, for, even if the neurones next upstream were internuncial neurones, axon reflexes in them would produce reflex discharges of motoneurones belonging to other muscles. Therefore it may be assumed that the antidromic impulse can travel as far as the motoneurone's synapses, but no further.

The reduction of the reflex response, *i.e.*, number of motoneurones discharging, by a preceding antidromic volley has been shown to be due to a raised neurone-threshold of the motoneurones.† The antidromic volley will give rise to a refractory period in the motoneurones, but it is necessary to consider whether an inhibitory process is also set up. Any such complicating inhibition, *e.g.*, by the axon collaterals of Golgi, seems most unlikely, because the effects of antidromic impulses in motor nerve fibres are confined to the motoneurones from which these fibres arise (*cf.* Denny-Brown, 1928, p. 328), and also because the duration of the raised neurone-threshold is much shorter than any known inhibitory process (*cf.* Samojloff and Kisseleff, 1927; Eccles and Sherrington 1931, *b*; 1931, *e*). Moreover preliminary investigations on the effects of two antidromic volleys on motoneurones show that the period of raised neurone-threshold set up by an antidromic volley is not altered by another antidromic volley which precedes it by more than  $5.5\sigma$ . At intervals less than this there

\* Antidromic impulses passing up those motor axons having collateral branches (the axon collaterals of Golgi) would presumably also traverse these collaterals. However, only a few motor axons are provided with collaterals, so it does not seem possible that impulses traversing them could be responsible for any of the results described in this paper, because all motoneurones have been affected similarly by antidromic impulses in their motor nerve fibres.

† This statement is not correct when the antidromic volley reaches the motoneurones after some of the centripetal impulses (see p. 1

is a slight increase in the duration of the raised neurone-threshold following the second antidromic volley, but this can be satisfactorily accounted for by the slowed rate of propagation of the second volley consequent on its travel (in the peripheral path) in the relatively refractory period following the first volley (Forbes, Ray and Griffith, 1923 ; Gasser and Erlanger, 1925). If the raised neurone-threshold set up by an antidromic volley were due to inhibition, a summation of the effects of successive antidromic volleys would be expected. The absence of such summation in the above experiments is strong evidence in favour of the raised neurone-threshold being due to a refractory period. Immediately following a single antidromic volley there is a short period during which the largest centripetal volley which can be employed is unable to evoke a reflex discharge. This is not strictly an absolutely refractory period, because the centripetal volley cannot be made indefinitely large. Then follows a period of gradual recovery until normal responsiveness is regained. This is a relatively refractory period.

The shortness of the path from the point of exit of the ventral root,  $v$ , to the tips of the dendrites of the motoneurones—not more than 4 mm. in all—indicates that the conduction-time is likely to be of such short duration as to be almost negligible. For the greater part of this path, *i.e.*, from  $v$  to the axon hillocks, the rate of propagation will not differ greatly from that in the motor fibres of peripheral nerve. As this distance is not greater than 2.5 mm.,  $0.04 \sigma$  should be a maximal value for the conduction-time. It is not possible to compute the conduction-time of the remainder of the path with any approach to accuracy ; but its total distance is so short that it would be very unlikely to have a significant duration. Experimental observations agree with these calculations as they show that the conduction-time through the spinal cord may be less than  $0.5 \sigma$  (Eccles and Sherrington, 1931, *a*).

Thus the values which have been obtained for the point  $v$  (fig. 3, *a*) can be applied to the synapses without any significant error. *Therefore the results on Table I show that, at the synapses, the duration of the relatively refractory period following an antidromic impulse is about  $10.5 \sigma$ , and that the upper limiting value for the absolutely refractory period is  $5.5 \sigma$  (both average values).*

In a later section (see p. 571) it will be shown that preformed c.e.s. is removed from a motoneurone by an antidromic impulse. Before a reflex discharge can occur after an antidromic impulse, it is therefore necessary for the c.e.s. to be built up to neurone-threshold. This will take some time (*cf.* Eccles and Sherrington, 1931, *a*) especially since the neurone-threshold would be higher than normal (on account of the relatively refractory period).

Thus the period of  $5.5\sigma$  is probably a generous upper limit to the duration of the absolutely refractory period. Facilitation between two centripetal volleys increases the rate of c.e.s. production (Eccles and Sherrington, 1931, *a*), consequently it should be possible to reduce the period of unresponsiveness following an antidromic volley by this means. Experimental observations confirm this deduction, *e.g.*, in experiment 17.7.30 the period of unresponsiveness was in this way reduced from  $5.3\sigma$  to  $3.9\sigma$ .

Gasser and Erlanger (1930) state that all the nerve fibres of the A group have the same absolutely refractory period, and that the  $\alpha$  and some of the  $\beta$  fibres have approximately the same relatively refractory period. Now the total duration of the refractory period for peripheral mammalian nerve is not more than  $5\sigma$ . The longer duration,  $10.5\sigma$ , found for the motoneurones (probably the synapses) may approximate more to the values for the B or C groups of peripheral nerve fibres (Erlanger and Gasser, 1930). If the absolutely refractory period of the "synaptic" region were also approximately double that of the A fibres of peripheral nerve, it would not have a duration of more than  $2\sigma$ . An absolutely refractory period of about this duration is indicated by experiments in which two antidromic volleys were set up at such a short interval apart that the second reached the "synaptic" region of the motoneurone only  $2.4\sigma$  after the first.

Only the following three factors can be responsible for the lengthening of the latent period by the antidromic volley.

1. *Slowed Conduction in the Motor Pathway.*—Owing to the short interval by which it is preceded by the antidromic volley, the reflex discharge may be travelling for the more central part of its course along nerve fibres in the relatively refractory state. Since  $5\sigma$  is the shortest interval which has been found between the antidromic volley and the reflex discharge (Table I, column 2), this factor will not affect conduction in the peripheral efferent pathway. Moreover the time of conduction along that part of the efferent pathway which lies within the spinal cord is so short (see p. 567) that no significant slowing can occur there. This factor, therefore, is of no importance in the lengthening of the latent period.

2. In order to explain several types of experimental observations it has been necessary to assume that the excitatory impulses incident on a motoneurone as the result of a single centripetal volley have often a considerable temporal dispersion (Eccles and Sherrington, 1931, *a*; 1931, *c*; 1931, *d*; see p. 579). Each excitatory impulse in turn adds to the c.e.s. of the motoneurone until neurone-threshold is reached. The time thus taken in building up c.e.s.

is responsible for most of the delay in the central reflex pathway (Eccles and Sherrington, 1931, *a*). Since the preceding antidromic volley raises the threshold of the motoneurones, more excitatory impulses are necessary to evoke a reflex discharge; hence the delay in setting up a reflex discharge is then longer than normal. Moreover the greater the increase in the threshold of the motoneurones, the longer will be the delay.

The lengthening of the latent period varies greatly from preparation to preparation, and this variation is probably related to the amount of temporal dispersion of the excitatory impulses incident on the motoneurones. The lengthening will, therefore, be likely to be greatest in those cases where the reflex discharges have the largest temporal dispersions. A comparison of columns 5 and 6, Table I, shows that this is not so when the temporal dispersion is measured by the method previously described (Eccles and Sherrington, 1931, *a*). But this method of measurement does not take any account of the temporal dispersion in those individual reflex arcs which have latent periods longer than the "modal" latent period. In experiment 14.7.30 the electrical response showed that a reflex discharge dispersed over several sigmata followed an initial almost synchronous discharge; hence the small value for temporal dispersion (Table I, column 6), although the latent period was lengthened as much as  $4.5\sigma$  by the antidromic volley.

3. In a later section it will be shown that preformed c.e.s. is removed from a motoneurone by an antidromic impulse. If an antidromic volley reaches the motoneurones after some of the earliest centripetal impulses, the c.e.s. produced by these impulses will be greatly reduced. Consequently the attainment of neurone-threshold by the c.e.s. will be delayed, and the reflex will have a longer latent period. In fig. 3 the earliest centripetal impulses  $c_1d_1$  reached the motoneurones after the latest antidromic volley  $a_3v_3$  which allowed a reflex discharge with a recognisable action-current to pass, and so this factor was not involved. This experiment is, however, exceptional in that respect. Usually a reflex response with a definite action-current is produced by a centripetal volley even when the earliest impulses reach the motoneurones  $2\sigma$  before the antidromic volley. The reflex discharge can only be produced by those excitatory impulses which arrive after the antidromic volley. The reflex discharge which occurs under these circumstances seems certain evidence that there is a temporal dispersion of the excitatory impulses incident on the motoneurones as the result of a single centripetal volley (*cf.* p. 579). Experiment 21.5.30 (Plate 39, fig. 1) is exceptional probably because there is a comparatively small temporal dispersion of the excitatory impulses incident on

the motoneurones. A similar explanation would account for the small lengthening of the latent period and the absence of any measurable temporal dispersion in this experiment (columns 5 and 6, Table I).

(iv) *Conclusions*.—When an impulse travels up a motor nerve fibre to the spinal cord (an antidromic impulse), it traverses the surface of the associated motoneurone with its dendrites. The ensuing raised neurone-threshold indicates that a central refractory period has been set up, and that its total duration is about  $10.5\sigma$ . The earlier part of this period is an absolutely refractory period having a duration not longer than  $5.5\sigma$  (most probably considerably shorter than this), the latter part is a relatively refractory period.

The reflex response which is not prevented by the antidromic volley has a longer latent period than normal. The temporal dispersion of the excitatory impulses incident on the motoneurones together with the raised neurone-threshold probably account for most of this lengthening. When the antidromic volley arrives after some of the centripetal impulses, it removes the c.e.s. formed by them and so delays the attainment of threshold in any motoneurones which still respond by a reflex discharge. This accounts for some of the lengthening of latent period when the antidromic volley is late relatively to the centripetal.

## 2. *The Effect of an Antidromic Volley on the Central Excitatory State.*

(i) *Introduction*.—A centripetal volley sets up an enduring condition called the central excitatory state, c.e.s., in the motoneurones of the subliminal fringe in addition to the reflex discharge which may or may not be evoked from other motoneurones (Eccles and Sherrington, 1930; 1931, *b*). The c.e.s. so produced can be detected by means of another similar centripetal volley following the first at a short interval. The c.e.s. persisting after the first volley sums with that produced by the second with the result that, in some motoneurones, the intensity of c.e.s. is sufficient to evoke a reflex discharge.

In order to investigate the effect, if any, which an antidromic volley has on the central excitatory state, it is necessary that this antidromic volley, A, should be timed so that it will reach the motoneurone after the production of c.e.s. by the first centripetal volley,  $C_1$ , and before the arrival of the second centripetal volley,  $C_2$ . When neither centripetal volley alone is able to evoke a reflex discharge, the results of the experiment are relatively easy to interpret and so will be considered first; but, unfortunately, in only one experiment has the facilitation between two such volleys been extensive enough to allow definite conclusions to be drawn. This is due to the difficulty of detecting the

small facilitated response when it is immediately preceded by the maximal motor twitch (a necessary consequence of the setting up of the antidromic volley). The conclusions of this single experiment have, however, been fully confirmed by the analysis of those experiments in which either centripetal volley alone produced a reflex discharge.

(ii) *Effect on the Facilitation between two Subliminal Centripetal Volleys.*—In Plate 39, fig. 2, the first observation shows the reflex response when the second subliminal centripetal volley  $C_2$  followed  $C_1$  by  $16.6 \sigma$ . In the other four observations the antidromic volley A was interposed at various intervals after the setting up of the first volley, the interval between  $C_1$  and  $C_2$  being kept constant. In the second and third observations both the electrical and mechanical responses show that the reflex response to  $C_1 + C_2$  is unaffected by A. In the fourth observation it suffers some reduction, while in the fifth observation the reflex response is still further reduced. The intervals between the setting up of  $C_1$ , A, and  $C_2$  were as follows :—

2nd observation	.....	$C_1$ $0.0 \sigma$ A $16.6 \sigma$ $C_2$
3rd	„	..... $C_1$ $3.2 \sigma$ A $13.4 \sigma$ $C_2$
4th	„	..... $C_1$ $6.3 \sigma$ A $10.3 \sigma$ $C_2$
5th	„	..... $C_1$ $9.5 \sigma$ A $7.1 \sigma$ $C_2$

The lengths of the peripheral nerve paths were not measured in this experiment; but judging by the position of the electrodes, and allowing 35 metres per second and 80 metres per second for the respective velocities of propagation of the centripetal and antidromic volleys (Eccles and Sherrington, 1931, *a*), it was calculated that the time taken by the *fastest* centripetal impulses to reach the motoneurones would be about  $5 \sigma$  longer than that taken by the antidromic impulses. Therefore the intervals between the arrival of the volleys at the motoneurones would be approximately :—

2nd observation	.....	A $5.0 \sigma$ $C_1$ $16.6 \sigma$ $C_2$
3rd	„	..... A $1.8 \sigma$ $C_1$ $16.6 \sigma$ $C_2$
4th	„	..... $C_1$ $1.3 \sigma$ A $15.3 \sigma$ $C_2$
5th	„	..... $C_1$ $4.5 \sigma$ A $12.1 \sigma$ $C_2$

In the second and third observations A reaches the motoneurones before  $C_1$ , so the absence of any effect on the c.e.s. produced by  $C_1$  is not surprising. The  $AC_2$  interval in the fourth and fifth records is so long that the central refractory period following A (see p. 567) cannot have any direct effect on the reflex

response to  $C_2$ . The reflex response must be reduced by reason of the diminution by A of the facilitating effect which  $C_1$  exerts on  $C_2$ .

Hence the evidence seems conclusive that the antidromic volley has been able to reduce greatly the c.e.s. which is produced by the first volley and which in many motoneurones sums with the c.e.s. produced by the second volley in order to give rise to a reflex discharge.

Many other series of observations similar to fig. 5 were also obtained in this experiment. When the  $C_1C_2$  interval was altered, the effect of A on the facilitated reflex response was found to be dependent on the interval by which A followed  $C_1$ . Thus when the stimulus-intervals were  $C_1$  4  $\sigma$  A 5.9  $\sigma$   $C_2$ , i.e., A 1  $\sigma$   $C_1$  9.9  $\sigma$   $C_2$  for the motoneurones, A had no effect on the facilitated reflex response, but this response was considerably reduced when the stimulus-intervals were  $C_1$  6.3  $\sigma$  A 6.3  $\sigma$   $C_2$ , i.e.,  $C_1$  1.3  $\sigma$  A 11.3  $\sigma$   $C_2$  for the motoneurones. These results confirm the previous conclusion that the refractory period following the antidromic volley is not the cause of the reduction of the reflex. *Therefore, when an antidromic impulse reaches a motoneurone, the experimental results indicate that it reduces the preformed c.e.s. in that motoneurone.*

(iii) *Effect on the Facilitation between the Subliminal Fringes of two Centripetal Volleys.*—Plate 39, fig. 3, illustrates part of a series from experiment 28.7.30 in which both  $C_1$  and  $C_2$  alone evoked a reflex discharge. The second and fifth observations show that there was a considerable facilitation of  $C_2$  by  $C_1$ , notwithstanding the relatively long interval (23.9  $\sigma$ ) between  $C_1$  and  $C_2$  (the response to  $C_2$  alone is shown by the fourth observation). In the third and sixth observations the antidromic volley A was set up as well as both the centripetal volleys, the stimulus-intervals being  $C_1$  19.8  $\sigma$  A 4.1  $\sigma$   $C_2$ , and the approximate intervals between the times of arrival at the motoneurones of the *fastest*  $C_1$  and  $C_2$  impulses and of the A impulses being  $C_1$  18.2  $\sigma$  A 5.7  $\sigma$   $C_2$ . The action-current of the small reflex response to  $C_1$  just preceded the large motor action-current, and this in turn was followed by a small ill-defined action-current (shown by the arrow). This was all that was left of the response to  $C_2$ . As a consequence of the antidromic volley, A, the large facilitated response to  $C_2$  had been reduced so that it was even less than the response to  $C_2$  alone.\* The central refractory period following A could not have been

\* A small amount of inhibition following  $C_1$  (Eccles and Sherrington, 1931, b) was probably responsible for this reduction to a value *less* than the response to  $C_2$  alone (*vide* also Plate 40, fig. 4). The effect of this inhibition would be normally masked by the preponderating amount of c.e.s. produced by  $C_1$ . Inhibition is unaffected by an antidromic

responsible for this great reduction, because control records showed that, at that  $AC_2$  interval, there was no appreciable reduction of the response to  $C_2$  when it was preceded only by A. This is not surprising because the earliest reflex discharge was evoked by  $C_2$  about  $12\sigma$  after A reached the spinal cord,\* i.e., at an interval longer than the usual duration of the relatively refractory period (see p. 567). The reduction must have been due to the removal by A of the facilitating influence which  $C_1$  exerted on  $C_2$ ; in other words A removed the c.e.s. which was produced by  $C_1$ .

Many other series of observations were made in experiment 28.7.30, which confirmed the observations of Plate 39, fig. 3, e.g., the first five observations of Plate 40, fig. 4. A similar series of observations was also made in experiment 23.7.30. In all these experiments the reduction of the reflex response evoked by  $C_2$  must have been due to the diminution by A of the facilitating influence of  $C_1$  on  $C_2$ . There is thus perfect agreement with the results of experiment 7.10.29.

In other experiments which have been performed the duration of the facilitating influence of  $C_1$  on  $C_2$  was so short, that, when A was interposed, the response to  $C_2$  occurred in the relatively refractory period following A. A careful series of controls was necessary in order to allow for the effect of the refractory period.

The results of a complete series are given in Table II and Plate 40, fig. 5, shows that part of the series from which the measurements of observation 4 were made. The values of columns 3 and 6 have been calculated by the method of maximum tension differences referred to on p. 561, and so are about 1.3 times too large. In calculating column 3 the response to  $C_1 + A$  has been subtracted from that to  $C_1 + A + C_2$ , and in column 6 the response to A (the maximal motor twitch) has been subtracted from that to  $A + C_2$ . The values of column 4 are calculated directly by subtraction of the maximum tension evoked by  $C_1$  from that evoked by  $C_1 + C_2$ , and so do not suffer an increase similar to the values of columns 3 and 6. Therefore the ratios of 1.25 and 1.27 in observations 1 and 4 (column 5) indicate that the reflex response produced by  $C_2$ , when preceded by  $C_1$ , was not appreciably altered in tension by the antidromic volley A. The significance of this and of the high value

volley (Eccles and Sherrington, 1931, *b*; 1931, *d*), so its presence first becomes apparent after an antidromic volley has removed the c.e.s. In most experiments the inhibition is so powerful that it is difficult to demonstrate any facilitation of  $C_2$  by  $C_1$  even when the  $C_1C_2$  interval is comparatively short, e.g.,  $10\sigma$ .

\* The central reflex-time was unusually long (over  $6\sigma$ ).



Table II.

1	2	3	4	5	6	7	8
Order of observations.	Stimulus intervals.	Maximum tension added by $C_2$ to $C_1$ + A = X grm.	Maximum tension added by $C_2$ to $C_1$ = Y grm.	X/Y.	Maximum tension added by $C_2$ to A = W grm.	Maximum tension of response to $C_2$ alone = Z grm.	W/Z.
3	$C_1$ 3.2 $\sigma$ A 3.2 $\sigma$ $C_2$	239 219					
		Av. 229	128	1.79	136	160	0.81
1	$C_1$ 4.8 $\sigma$ A 0.0 $\sigma$ $C_2$	128 126 156	110 110		134 150		
		Av. 137	Av. 110	1.25	Av. 142	124	1.15
4	$C_1$ 4.8 $\sigma$ A 1.6 $\sigma$ $C_2$	122	96	1.27	142	162	0.88
2	$C_1$ 6.4 $\sigma$ A 0.0 $\sigma$ $C_2$	24 30 6 16	122 102		162 166	156 160	
		Av. 19	Av. 112	0.17	Av. 164	Av. 158	1.04
5	$C_1$ 6.4 $\sigma$ A 1.6 $\sigma$ $C_2$	8 32					
		Av. 20	100	0.20	110	154	0.72

1.79 of observation 3 will be discussed on p. 581. In observations 2 and 5, on the other hand, the values 0.17 and 0.20 in column 5 show that A has effected a great reduction in the reflex response to  $C_2$  when preceded by  $C_1$ . The possibility of this being due to a refractory period following A will be dealt with first.

The control observations of columns 6, 7 and 8 show that, when  $C_2$  was not preceded by  $C_1$ , A reduced the response to  $C_2$  by a comparatively small amount. At the  $AC_2$  stimulus-intervals employed this is in agreement with previous results (see p. 562). Still it must be remembered that the latent period of the response to  $C_2$  was shortened when it was preceded by  $C_1$  (Eccles and Sherrington, 1931, *a*), so the values of columns 6, 7 and 8 would not be reliable controls. However, the X/Y values (column 5) give clear evidence that, when following  $C_1$ , the effect of A on  $C_2$ , while almost independent of the  $AC_2$  interval,\* is

\* This is only true for relatively small changes in the  $AC_2$  interval. When the alteration is considerable, e.g., 10  $\sigma$ , the  $AC_2$  interval is more important (see p. 576).

intimately related to the  $C_1$  A interval. In Table III the values of column 5, Table II, are arranged to show this.

Table III.—Values of X/Y (column 5, Table II) arranged according to the Stimulus-intervals,  $C_1$  A (rows), and A  $C_2$  (columns).

—	A 0 $C_2$ .	A 1.6 $\sigma$ $C_2$ .	A 3.2 $\sigma$ $C_2$ .
$C_1$ 3.2 $\sigma$ A .....	—	—	1.79
$C_1$ 4.8 $\sigma$ A .....	1.25	1.27	—
$C_1$ 6.4 $\sigma$ A .....	0.17	0.20	—

This result furnishes strong evidence that the response to  $C_2$  has been indirectly affected through the action of A on the subliminal fringe of  $C_1$ , and not directly by the refractory period following A. In view of the definite findings of experiments 7.10.29, 23.7.30, and 28.7.30, it can be concluded that, in these observations also, the antidromic volley A removed the c.e.s. formed by the precurent centripetal volley  $C_1$  when the  $C_1$  A interval was sufficiently long. The details of the temporal relationships between  $C_1$ , A and  $C_2$  will be considered on p. 581. This conclusion was confirmed by similar series of observations in six other experiments.

(iv) *Conclusions.*—Thus the experimental results on the subliminal fringe agree with experiment 7.10.29 in showing that an antidromic impulse can greatly reduce the c.e.s. of the motoneurone into which it is fired. It would be difficult to prove experimentally that there was a complete removal of all the c.e.s.; but, in such cases as fig. 6, the amount of c.e.s. left must have been vanishingly small, for none was revealed by testing with the second centripetal volley  $C_2$ . Experiment has repeatedly shown a reduction of c.e.s. which is almost, if not quite, complete, and in no experiment has a large reduction not been found. On general grounds it seems improbable that an antidromic impulse would remove only part of the c.e.s. of a motoneurone. If it removed any, it would be likely to remove all, i.e., its action on the c.e.s. of a motoneurone would be likely to have an all-or-nothing character. *Thus from present evidence it seems that, when an antidromic impulse is fired into a motoneurone, there is a removal of the preformed c.e.s. of that motoneurone.*

Since in some cases, e.g., Plate 39, fig. 3, all the facilitating influence of one centripetal volley on another can be prevented by an antidromic volley, it follows that in these cases the c.e.s. responsible for such facilitation cannot be further upstream than the "synapses" of the motoneurones, for these are

the limits to the passage of the antidromic volley (see p. 566). Because in these cases no c.e.s. appears to exist at any region other than the "synapses" of the motoneurones, it seems probable that there are no other "synapses" in the reflex pathway. If that is so, there are no internuncial neurones, and the terminal branches of the afferent nerve fibres make direct contact with the dendrites and perikaryon of the motoneurone by means of synapses, i.e., the flexor reflex will be an example of the simplest type of reflex arc (Sherrington, 1906). It will be seen later that the flexor reflex arc is probably not always of this simple type; for the internuncial neurone is the most likely explanation of certain observations (see p. 579).

### 3. The Time-factor in the Formation of Central Excitatory State.

(i) *Experimental Observations.*—In the sixth record of Plate 40, fig. 4, the stimulus-intervals were  $C_1$  7.9  $\sigma$  A 16  $\sigma$   $C_2$ . The response to  $C_2$  gave evidence that the facilitation of it by  $C_1$  was unaffected by A. Now the intervals between the arrival at the spinal cord of the *foremost* impulses of the respective centripetal volleys and the antidromic volley were approximately  $C_1$  6.2  $\sigma$  A 17.7  $\sigma$   $C_2$ . Thus A was unable to affect the facilitation of  $C_2$  by  $C_1$  when it arrived as much as 6  $\sigma$  later than the foremost impulses of  $C_1$ .

The complete series of observations is shown in Table IV, the intervals being for the times of arrival of the *fastest* impulses at the spinal cord (this will closely approximate to the times of arrival at the motoneurones (see p. 567)).

Table IV.

Order of observation.	Intervals at spinal cord.*	$\frac{\text{Maximum tension added by } C_2 \text{ to } C_1 + A}{\text{Maximum tension added by } C_2 \text{ to } C_1} = \frac{X}{Y}$
2	$C_1$ 6.2 $\sigma$ A 17.7 $\sigma$ $C_2$	1.02 (average of 2)
3†	$C_1$ 6.2 $\sigma$ A 17.7 $\sigma$ $C_2$	0.99 (average of 2)
4	$C_1$ 12.1 $\sigma$ A 11.8 $\sigma$ $C_2$	0.87
1	$C_1$ 18.1 $\sigma$ A 5.8 $\sigma$ $C_2$	0.03 (average of 3)
5	$C_1$ 22.2 $\sigma$ A 1.7 $\sigma$ $C_2$	0.11 (average of 2)

\* It is important to remember that in this and all other cases the intervals given for the spinal cord refer to the times of arrival of the fastest centripetal impulses and not to the reflex discharge. This does not take place till later (the delay accounts for most of the central reflex-time). The reduction of the reflex owing to the central refractory period set up by the antidromic volley is determined by the interval at which the *reflex discharge* follows the antidromic volley (see Table I). Since in this case the central reflex-time was more than 7  $\sigma$ , the effect of refractory period would be small in observation 5 and absent in all the other observations of Table IV.

†  $C_1$  and  $C_2$  both stronger than for observation 2.

Later in the experiment when the intervals at the spinal cord were  $C_1$   $6.2 \sigma$  A  $4.9 \sigma$   $C_2$ , a value of 0.20 was obtained for X/Y instead of 1.02 and 0.99 given for a similar  $C_1$  A interval in Table IV, and when the intervals were  $C_1$   $4.2 \sigma$  A  $7.6 \sigma$   $C_2$ , a value of 0.17 was obtained. It seems that the alteration in the A  $C_2$  interval was responsible for the difference between these values and those of Table IV with approximately similar  $C_1$  A intervals.

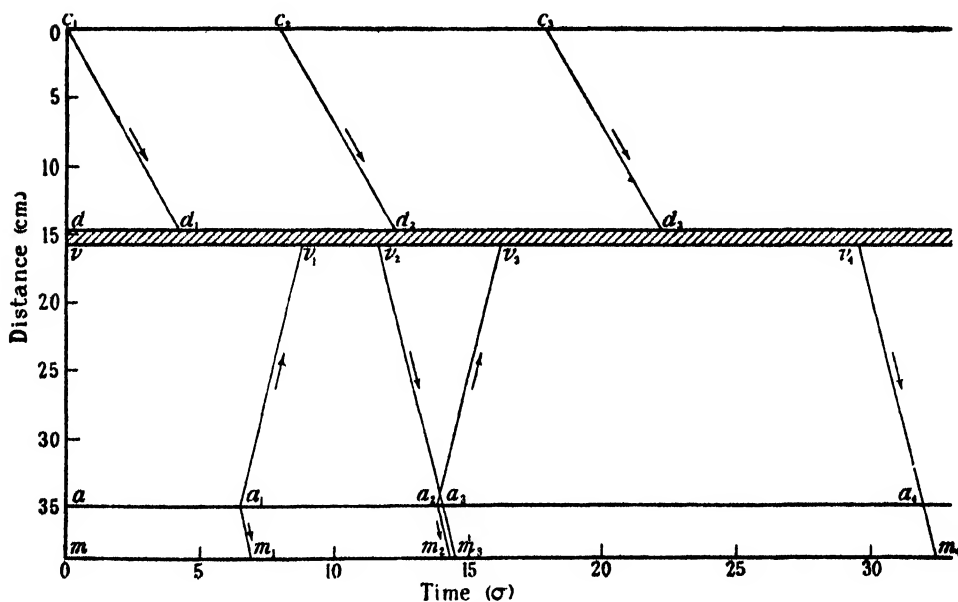


Fig. 4.

Observations made in experiment 23.7.30 after those in Table II indicate a similar effect. Plate 40, fig. 6, shows part of the series, and the results are drawn to scale in fig. 4 (*cf.* fig. 3),  $c_1d_1$  and  $c_2d_2$  representing  $C_1$  and  $C_2$  respectively. The reflex response to  $c_1d_1$  (shown by  $v_2a_2m_2$ ) had a central reflex-time of more than  $7\sigma$ , and even when it followed  $c_1d_1$  the central reflex-time of the response to  $c_2d_2$  (shown by  $v_4a_4m_4$ ) was still over  $7\sigma$ . Since the  $c_1c_2$  interval was  $17.9\sigma$ , the response evoked by  $c_2d_2$  probably would not be affected by any

refractory period following the response to  $c_1d_1$ , and in this experiment there was no evidence of any inhibition following  $c_1d_1$  (Eccles and Sherrington, 1931, b); therefore all the motoneurons which responded to  $c_3d_3$  alone would also respond when it was preceded by  $c_1d_1$ . When this is allowed for, both the electrical and mechanical records in the first three observations of Plate 40, fig. 6, show that  $c_1d_1$  exerted a considerable facilitating influence on  $c_3d_3$ .

When an antidromic volley  $a_1v_1$  was set up 6.4  $\sigma$  after  $C_1$  (*vide* sixth observation, Plate 40, fig. 6), the large action-current of the response to  $c_3d_3$  was identical with the response to  $c_3d_3$  when it was preceded by  $c_1d_1$  only (*vide* second and third observations, Plate 40, fig. 6). This is confirmed by the mechanical records, for the method of maximum differences gives a value of 1.31 for X/Y (calculated as in Table II, column 5). Therefore  $a_1v_1$  has no effect on the facilitation by  $c_1d_1$  of the response to  $c_3d_3$ . But the immediately preceding observations shown in Table II, observation 5, indicate that, when  $C_2$  (represented by  $c_2d_2$  in fig. 4) was set up 8  $\sigma$  after  $C_1$ , the antidromic volley  $a_1v_1$  was able to remove almost all of the facilitating effect of the first centripetal volley on the second. Thus those observations agree with the results of experiment 28.7.30 in showing that an antidromic volley reduced the facilitating effect of  $C_1$  on  $C_2$  only when the A  $C_2$  interval was comparatively short.

In the fourth observation of Plate 40, fig. 6, the antidromic volley was set up 13.8  $\sigma$  after  $C_1$  (it is shown by  $a_2v_2$  in fig. 4), while the second centripetal volley  $c_3d_3$  still followed  $c_1d_1$  by 17.9  $\sigma$ . The response to  $c_3d_3$  is shown by its action-current to be considerably diminished. This is confirmed by the mechanical record which gives a value of 0.84 for X/Y (average of two determinations) instead of 1.31 which was obtained when there was no diminution of the response to  $c_3d_3$ . This value of 0.84 does not allow for the response which was evoked by  $c_3d_3$  alone; this latter should suffer no reduction by the refractory period following  $a_2v_2$ , because the interval between  $v_2$  and  $v_4$  was more than 13  $\sigma$  (see p. 567). Since X/Y has a value of about 0.6 for that part of the response to  $c_3d_3$  which was due to facilitation by  $c_1d_1$ , more than half of this facilitation must have been prevented by the later antidromic volley  $a_2v_2$ , although the earlier volley  $a_1v_1$  was entirely ineffective.\*

\* The centrifugal reflex impulses (shown by  $v_4a_4$ ) would prevent the antidromic volley from reaching those motoneurons which responded to  $c_1d_1$  alone. Since the centripetal volley  $c_3d_3$  was similar to  $c_1d_1$  in strength, the motoneurons which responded to  $c_1d_1$  alone also responded to  $c_3d_3$  alone, and so were not concerned in the facilitation by  $c_3d_3$  of  $c_1d_1$ . The blocking of the antidromic impulses to them was therefore immaterial.

(ii) *Discussion.*—The difference in behaviour between the earlier and later antidromic volleys in experiments 23.7.30 and 28.7.30 is most easily explained by the following hypothesis.

The formation of c.e.s. by a centripetal volley is a process which continues for some time, probably owing to a considerable temporal dispersion in the times of arrival of the excitatory impulses. This temporal dispersion will be a consequence partly of different conduction-times of the centripetal impulses in the peripheral afferent nerve, partly of different conduction-times in the central reflex pathways (the delay-paths of Forbes (1922)), *e.g.*, due to internuncial neurones. From certain observations it will be inferred (see p. 580) that the c.e.s. produced by any particular impulse persists for some time as a state of gradually diminishing intensity. If that is so, then the total intensity of the c.e.s. present in a motoneurone at any particular instant will be made up, partly of the c.e.s. which is just being produced by excitatory impulses, and partly of the c.e.s. which has been produced by previous impulses, and which still persists (*cf.* Eccles and Sherrington, 1931, *a*).

Thus following  $d_1$  (fig. 4), the point of arrival of the foremost centripetal impulse  $c_1d_1$  set up by the stimulus at  $c_1$ , there would be a period during which the motoneurones were subjected to a continuous bombardment by excitatory impulses. In accordance with the previous conclusions the antidromic volley  $a_1v_1$  removed the c.e.s. which had been formed, and very little c.e.s. had been produced by fresh impulses at the time of the arrival of the impulses of the second centripetal volley  $c_2d_2$ ; hence the almost complete absence of facilitation as shown by the small X/Y ratio (0.20). When, however, the second centripetal volley (shown by  $c_3d_3$ ) followed  $a_1v_1$  at a longer interval, the c.e.s. had almost been built up to a normal intensity by the excitatory impulses which had arrived after  $v_1$ ; hence the facilitation of  $c_3d_3$  by  $c_1d_1$  was unaffected by  $a_1v_1$ . For that to be so, no appreciable amount of the c.e.s. present at  $v_1$  could normally have persisted until  $v_4$ . But when the antidromic volley was set up later as shown at  $a_2v_2$ , the c.e.s. was not built up to a normal intensity after its complete removal at  $v_2$ ; hence the facilitation of  $c_3d_3$  was diminished by  $a_2v_2$ . Some of the c.e.s. already formed at  $v_2$  must normally have persisted and helped to facilitate the response to  $c_3d_3$  ( $v_4a_4m_4$ ).

The only alternative explanation would involve the supposition of a restoration of c.e.s. which had been rendered ineffective by the antidromic volley. There is no evidence to justify this assumption. On general grounds a process of this type, distinct from refractory period, is most improbable. Moreover the first hypothesis is more in agreement with experiments on after-discharge

(Eccles and Sherrington, 1931, c), and it offers a satisfactory explanation both of the temporal dispersion of the reflex discharge (Eccles and Sherrington, 1931, a), and of the shortening of the latent period of a reflex by increasing the strength of the centripetal volley which evokes it (Eccles and Sherrington 1931, a). In addition no other hypothesis seems to be able to explain satisfactorily the observations described in the next section (see p. 581).

(iii) *Conclusions.*—*Thus it seems likely that a single centripetal volley can give rise to c.e.s. during a considerable period, and that in any motoneurone an antidromic impulse permanently removes the c.e.s. which it finds preformed.*

The facilitation of the response to a centripetal volley by a preceding centripetal volley is evidence that some c.e.s. formed by the earlier volley was present when the response to the second volley was set up. There are three possible causes of this :—

- (1) The c.e.s. produced by any particular impulses itself enduring for some time (*cf.* Eccles and Sherrington, 1930, a).
- (2) The continued formation of c.e.s. due to impulses of the first volley continually arriving.
- (3) Both of these processes operating together.

The above hypothesis favours the third cause, and makes it possible to give approximate values to the durations of both of the processes concerned. For example it has been already deduced (see p. 579) that c.e.s. persisted for an interval as long as  $v_3v_4$  (fig. 4), *i.e.*, for about  $13\sigma$ . By a similar calculation it is found that some of the c.e.s. in observation 4, Table IV, persisted for about  $19\sigma$ . Again the antidromic volley  $a_2v_3$  did not prevent all the facilitation by  $c_1d_1$  of  $c_3d_3$  (*vide* note, p. 578), so some of the delayed impulses set up by  $C_1$  must have arrived after  $v_3$ , *i.e.*, at least  $12\sigma$  after the first impulses at  $d_1$ ; therefore the temporal dispersion of the excitatory impulses due to a single centripetal volley must be greater than  $12\sigma$ . Observation 4, Table IV, leads to a similar conclusion in experiment 28.7.30.

All the conclusions arrived at in this section on the time-factor in the formation of c.e.s. have been based on the observations of only two experiments, 23.7.30 and 28.7.30. In only these experiments was there facilitation at a sufficiently long interval between the centripetal volleys. In other experi-

ful inhibition as well as excitation (Eccles and Sherrington, 1931, b). This inhibition was probably entirely responsible for the absence of facilitation at long intervals. In experiments 23.7.30 and 28.7.30 inhibition was never

strong enough to be significant, and so the observations of these experiments probably give a more reliable idea of an uncomplicated condition of c.e.s. production than do other experiments.

4. *The Time-factor in the Action of an Antidromic Volley on the Central Effect of a Centripetal Volley.*

(i) *Discussion.*—The conditions prevailing during the series of observations shown in Table II are diagrammatically represented in fig. 5 according to the

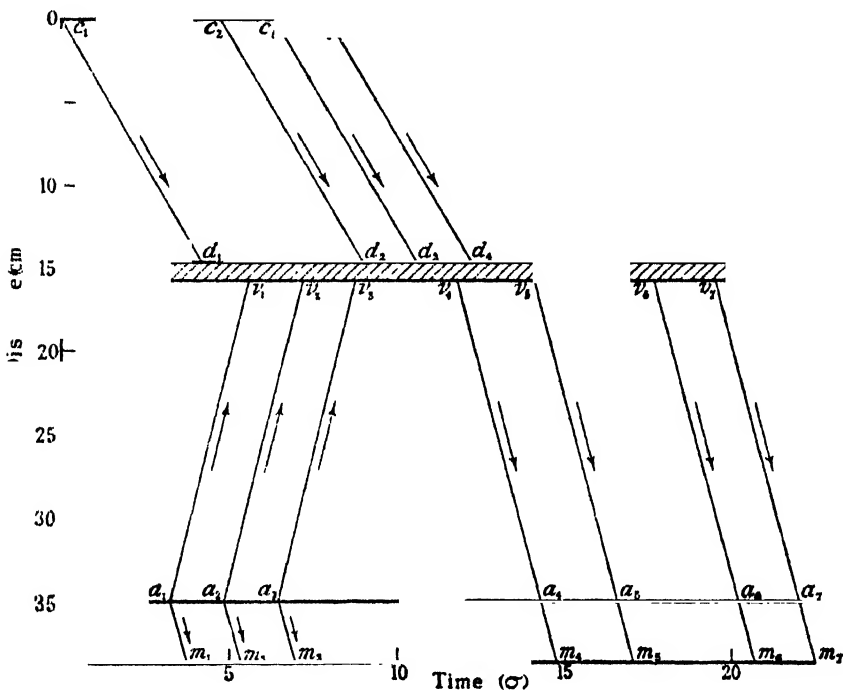


FIG. 5.

usual convention. The second centripetal volley  $C_2$  is represented by  $c_2d_2$ , or  $c_3d_3$ , or  $c_4d_4$  according as the  $C_1C_2$  interval was  $4.8\sigma$ , or  $6.4\sigma$ , or  $8.0\sigma$ , and the antidromic volley A is represented by  $a_1v_1$ , or  $a_2v_2$ , or  $a_3v_3$  according as the  $C_1A$  interval was  $3.2\sigma$ , or  $4.8\sigma$ , or  $6.4\sigma$ . When the stimulus-intervals were  $C_1$   $3.2\sigma$  A  $3.2\sigma$   $C_2$  the value of X/Y was 1.79 (observation 3, Table II). This high value is certain evidence that not only was the facilitation by  $C_1$  of  $C_2$  ( $c_2d_2$  in fig. 5) not diminished by A ( $a_1v_1$  in fig. 5), but it was actually considerably increased, i.e., A has increased the number of motoneurones in the



effective subliminal fringe\* following  $C_1$ . The following explanation of this result assumes that there is a temporal dispersion of the excitatory impulses incident on a motoneurone (see p. 579; Eccles and Sherrington, 1931, *d*, p. 599).

Experiment showed that the antidromic volley  $a_1v_1$ , though reaching the motoneurones later than the foremost impulses  $c_1d_1$  of the first centripetal volley  $C_1$ , only reduced the reflex response  $v_2a_2m_2$  evoked by  $C_1$  to about half. Those motoneurones which still reflexly responded to  $C_1$  must have done so by virtue of the delayed excitatory impulses which were incident on them after  $v_1$  (see p. 579). But the motoneurones which were prevented by  $a_1v_1$  from responding to  $C_1$  must also have been bombarded by delayed excitatory impulses after  $v_1$ , and so there would be a considerable accumulation of c.e.s. before the impulses of the second centripetal volley,  $C_2$ , arrived ( $d_2$  shows the arrival of the earliest impulse of  $C_2$ ); in other words the effective subliminal fringe produced by  $C_1$  would be increased by the addition of these motoneurones. However, some of the motoneurones of the effective subliminal fringe, produced by  $C_1$  alone, would be removed from that fringe by reason of the destruction of preformed c.e.s. brought about by  $a_1v_1$  in all the motoneurones of the reflex centre. The value of 1.79 for  $X/Y$  shows that the net result of these two factors was a considerable increase in the effective subliminal fringe. Owing to its short central reflex-time (Eccles and Sherrington, 1931, *b*), the facilitated response ( $v_4a_4m_4$  in fig. 5) evoked by  $c_1d_1 + c_2d_2$  actually occurred before the response  $v_2a_2m_2$  normally evoked by  $c_1d_1$  alone (this latter had an exceptionally long central reflex-time).

When the stimulus intervals were  $C_1$  4.8  $\sigma$  A 0.0  $\sigma$   $C_2$  ( $C_2$  shown by  $c_2d_2$  in fig. 5) and  $C_1$  4.8  $\sigma$  A 1.6  $\sigma$   $C_2$  ( $C_2$  shown by  $c_2d_2$  in fig. 5), the respective values of  $X/Y$  were 1.25 and 1.27 (observations 1 and 4, Table II). Since  $X$  was calculated by the method of maximum tension differences (see p. 561), these values indicate that the effective subliminal fringe following  $C_1$  was practically unaltered by A ( $a_2v_2$  in fig. 5) when the  $C_1$ A interval was 4.8  $\sigma$ . At this interval A prevented almost all reflex response to  $C_1$  alone, i.e., the delayed impulses of  $C_1$  arriving after  $v_2$  were able to evoke a reflex discharge from only a few motoneurones. The remainder of those motoneurones normally excited to discharge by  $C_1$  alone were probably added to the effective subliminal fringe, and must have almost counter-balanced the diminution by  $a_2v_2$  of the motoneurones normally in this fringe. The resulting reflex dis-

\* This term signifies those motoneurones of the subliminal fringe produced by one centripetal volley, which can be excited to respond by a second similar centripetal volley at a suitable interval after the first (Eccles and Sherrington, 1931, *b*).

charges in observations 1 and 4 are shown by  $v_6a_6m_6$  and  $v_7a_7m_7$  respectively, i.e., so late as to be practically beyond the effect of the refractory period following  $a_3v_3$ .

When A ( $a_3v_3$  in fig. 5) was set up  $6.4\sigma$  after  $C_1$  the low X/Y values, 0.17 and 0.20, of observations 2 and 5, Table II, indicate that there was a great reduction in the effective subliminal fringe following  $C_1$ . The motoneurones were subjected to the effect of A at a time so late relatively to  $C_1$ , that not only was the reflex discharge evoked by  $C_1$  prevented, but also the effective subliminal fringe produced by it was considerably diminished.

Series of observations similar to those of experiment 23.7.30 were also obtained in the four other experiments in which similar observations were made. In all the X/Y value indicated that the effective subliminal fringe produced by a centripetal volley was larger than normal when an antidromic volley was set up so as to reach the motoneurones about  $1.5\sigma$  after the foremost centripetal impulses.

(ii) *Conclusion.*—Thus the temporal dispersion of the excitatory impulses incident on the motoneurones affords a satisfactory explanation of the variations in the effect produced by an antidromic volley on the facilitation between two centripetal volleys when the temporal relationships between all three volleys are altered.

#### SUMMARY.

When a single stimulus is applied to an intact motor nerve, a volley of impulses (called an antidromic volley) passes into the spinal cord through the ventral roots. If all the afferent nerve fibres in the motor nerve are cut centrally, it is possible to investigate the effect of this antidromic volley on the motoneurones which it reaches.

There has never been any evidence that an antidromic volley evokes a reflex discharge from motoneurones.

The antidromic volley sets up a condition of raised neurone-threshold in the reflex centre which lasts for about  $10.5\sigma$ . Evidence is presented which shows that this raised neurone-threshold is entirely due to a central refractory period. The duration of the absolutely refractory period is less than  $2.5\sigma$ , while the remaining  $8\sigma$  is a relatively refractory period.

If an antidromic volley is timed so that it reaches the motoneurones between two centripetal volleys, it greatly reduces or abolishes any facilitating influence which the first centripetal volley may exert on the second, provided that the second centripetal volley does not follow the antidromic volley by too long an interval (the effect of the raised neurone-threshold set up by the antidromic

volley has been excluded). In these latter cases it seems likely that the facilitation which is not removed by the antidromic volley is due to impulses of the first centripetal volley which arrive after the antidromic volley, *i.e.*, there is a considerable temporal dispersion of the excitatory impulses. Many other experimental observations also indicate that there is a temporal dispersion of the excitatory impulses incident on the motoneurons as the result of a single centripetal volley.

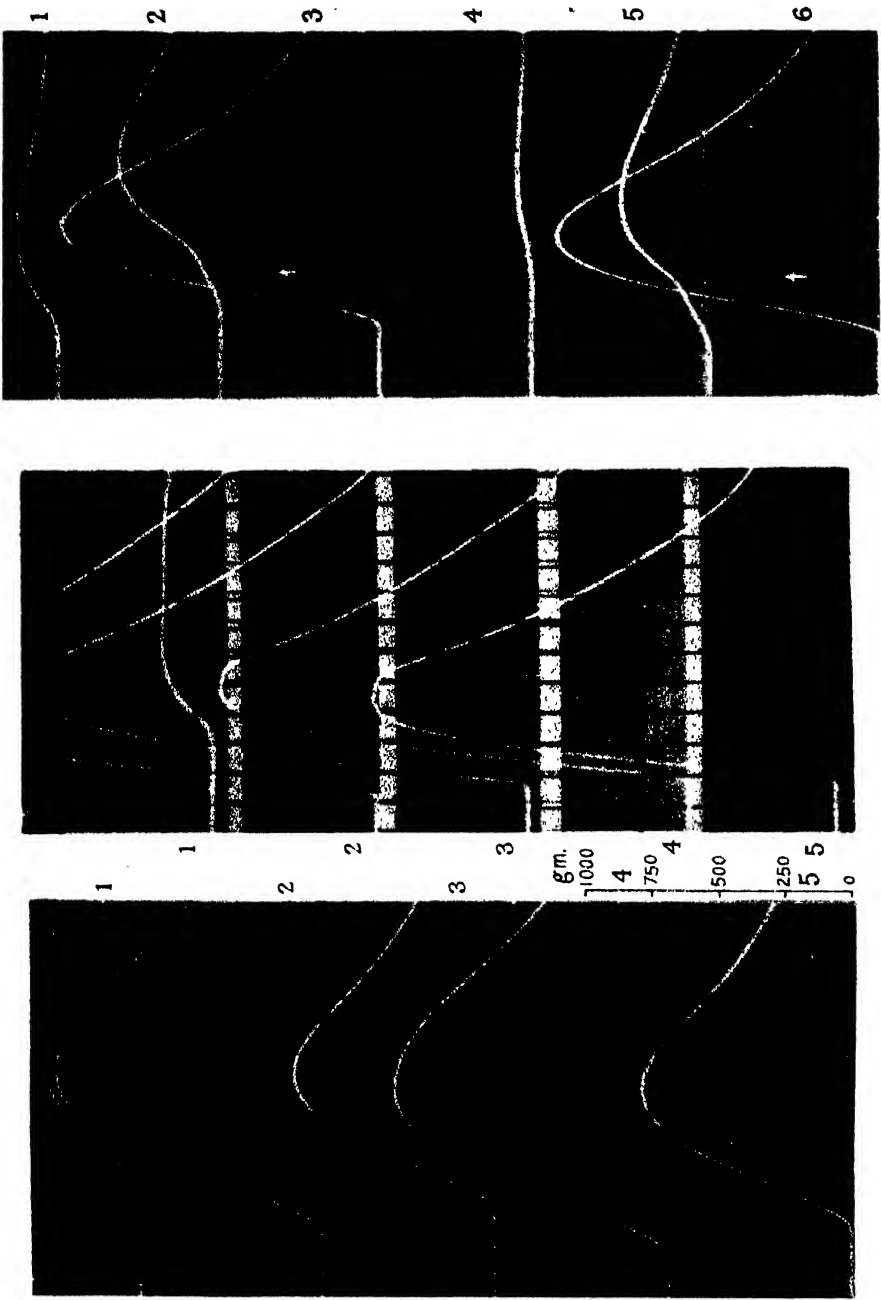
From the experimental evidence it is therefore concluded :—

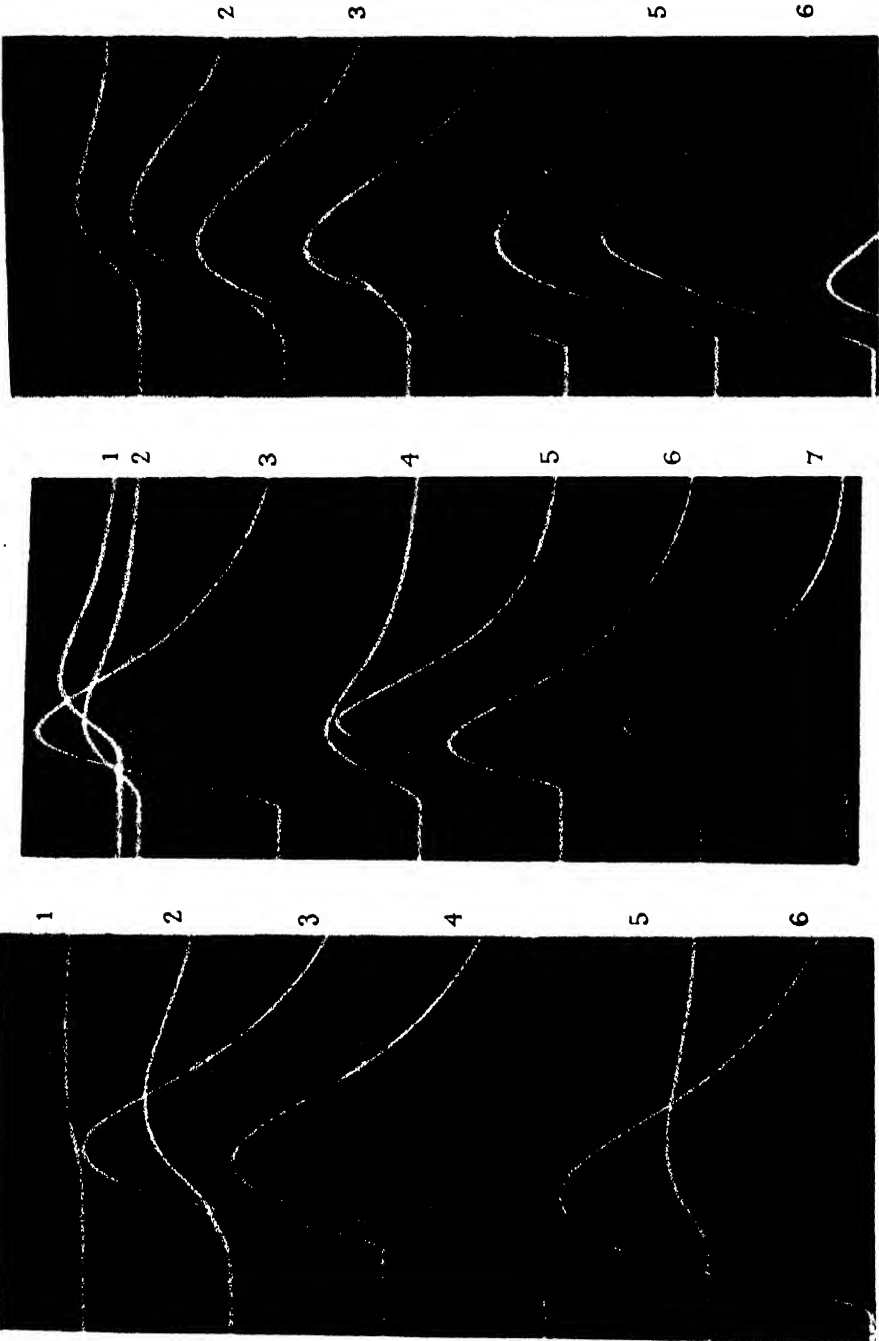
- (a) that a single centripetal volley gives rise to c.e.s. during a considerable period ;
- (b) that the persistence of the c.e.s. set up by a single centripetal volley is due partly to the temporal dispersion of the incident excitatory impulses and partly to the c.e.s. produced by any particular impulse itself enduring for some time ; and
- (c) that in any motoneurone an antidromic impulse removes the c.e.s. which it finds preformed.

I wish to express my thanks to Sir Charles Sherrington for his valuable help in all parts of this research. I also have to thank the Medical Research Council for a personal grant, and the Christopher Welch Trustees for defraying in part the expense of the photographic material.

#### REFERENCES.

- Bayliss, W. M. (1901). 'J. Physiol.,' vol. 26, p. 173.  
 Bernstein, J. (1898). 'Pflügers Archiv,' vol. 73, p. 374.  
 Cajal, Ramon y (1891). 'Rev. Ciencias Méd. Barcelona,' p. 1.  
 Denny-Brown, D. E. (1928). 'Proc. Roy. Soc.,' B, vol. 103, p. 321.  
 Denny-Brown, D. E. (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 252.  
 Eccles, J. C., and Hoff, H. E. (1931). As yet unpublished observations.  
 Eccles, J. C., and Sherrington, C. S. (1930). 'J. Physiol.,' vol. 69, p. 1.  
 Eccles, J. C., and Sherrington, C. S. (1931, a). 'Proc. Roy. Soc.,' B, vol. 107, p. 511.  
 Eccles, J. C., and Sherrington, C. S. (1931, b). 'Proc. Roy. Soc.,' B, vol. 107, p. 535.  
 Eccles, J. C., and Sherrington, C. S. (1931, c). 'Proc. Roy. Soc.,' B, vol. 107, p. 586.  
 Eccles, J. C., and Sherrington, C. S. (1931, d). 'Proc. Roy. Soc.,' B, vol. 107, p. 597.  
 Eccles, J. C., and Sherrington, C. S. (1931, e). In the course of publication.  
 Erlanger, J., and Gasser, H. S. (1930). 'Amer. J. Physiol.,' vol. 92, p. 43.  
 Forbes, A. (1922). 'Phys. Rev.,' vol. 2, p. 361.  
 Forbes, A., and Gregg, A. (1915). 'Amer. J. Physiol.,' vol. 37, p. 118.  
 Forbes, A., Ray, L. H., and Griffith, F. R. (1923). 'Amer. J. Physiol.,' vol. 66, p. 553.  
 Gasser, H. S., and Erlanger, J. (1925). 'Amer. J. Physiol.,' vol. 73, p. 613.  
 Gasser, H. S., and Erlanger, J. (1930). 'Amer. J. Physiol.,' vol. 94, p. 247.  
 Gerard, R. W., and Forbes, A. (1928). 'Amer. J. Physiol.,' vol. 86, p. 186.





- van Gehuchten, A. (1900). "Anatomie du Système Nerveux de l'Homme," p. 212.  
 Hoffman, P., and Keller, C. (1928). 'Z. Biol.,' vol. 87, p. 527.  
 Magandie, F. (1922). 'J. Physiol., expér. et path.,' vol. 2, p. 366.  
 Mialowski, N. (1895). 'C. R. Soc. Biol.,' Paris, p. 488.  
 Samojloff, A., and Kisseleff, M. (1927). 'Pflügers Arch.,' vol. 215, p. 699.  
 Sherrington, C. S. (1897). 'Proc. Roy. Soc.,' B, vol. 61, p. 243.  
 Sherrington, C. S. (1900). 'Sharpey-Schafer's Text-book of Physiology,' vol. 2, p. 798.

## EXPLANATION OF PLATES.

## PLATE 39.

FIG. 1.—Electrical (dark lines) and mechanical (white lines) records of responses of tibialis anticus muscle to a single centripetal volley set up in the posterior tibial nerve at various times relative to the application of a single stimulus to the intact motor nerve (peroneal). The stimulus-intervals were as follows (C and A denote the stimuli to afferent and motor nerves respectively). Observation 1, C alone. Observation 2, A 1  $\sigma$  C. Observation 3, C 7.9  $\sigma$  A. Observation 4, A 6.4  $\sigma$  C. Observation 5, C 5.9  $\sigma$  A. Time, 1 d.v. = 10  $\sigma$ . The resting position of the myograph record is below the corresponding electrical record in this and all subsequent figures. Tension scale at side.

FIG. 2.—Electrical and mechanical responses of tibialis anticus muscle evoked by two centripetal volleys  $C_1$  and  $C_2$  (either alone subliminal) set up in posterior tibial nerve at various times relative to a stimulus applied to the intact motor nerve (peroneal). The intervals between the stimuli were as follows:—1,  $C_1$  16.6  $\sigma$   $C_2$ . 2,  $C_1$  0.0  $\sigma$  A 16.6  $\sigma$   $C_2$ . 3,  $C_1$  3.2  $\sigma$  A 13.4  $\sigma$   $C_2$ . 4,  $C_1$  6.3  $\sigma$  A 10.3  $\sigma$   $C_2$ . 5,  $C_1$  9.5  $\sigma$  A 7.1  $\sigma$   $C_2$ . A myograph with a "friction" bearing was employed for the mechanical record and so the responses show "angles." Intervals between the vertical lines = 20  $\sigma$ .

FIG. 3.—Electrical and mechanical responses of tibialis anticus muscle. The centripetal volleys were set up in popliteal nerve. The observations were as follows:—1,  $C_1$  alone (electrical record not shown). 2,  $C_1$  23.9  $\sigma$   $C_2$ . 3,  $C_1$  19.8  $\sigma$  A 4.1  $\sigma$   $C_2$ . 4,  $C_2$  alone. 5,  $C_1$  23.9  $\sigma$   $C_2$ . 6,  $C_1$  19.8  $\sigma$  A 4.1  $\sigma$   $C_2$ . Tension scale as for Plate 39, fig. 1.

## PLATE 40.

FIG. 4.—As in fig. 3, Plate 39, later in the same experiment. The observations were as follows:—1,  $C_2$  alone. 2,  $C_1$  23.9  $\sigma$   $C_2$ . 3, A 4.1  $\sigma$   $C_1$ . 4,  $C_1$  19.8  $\sigma$  A 4.1  $\sigma$   $C_2$ . 5,  $C_2$  alone. 6,  $C_1$  7.9  $\sigma$  A 16  $\sigma$   $C_2$ . 7, A alone. Time, 1 d.v. = 10  $\sigma$ . Tension scale as for Plate 39, fig. 1.

FIG. 5.—As in fig. 3, Plate 39, in another experiment. The observations were as follows:—1,  $C_2$  alone (no electrical record). 2,  $C_1$  alone. 3, A 1.6  $\sigma$   $C_2$ . 4,  $C_1$  6.4  $\sigma$   $C_2$ . 5,  $C_1$  4.8  $\sigma$  A. 6,  $C_1$  4.8  $\sigma$  A 1.6  $\sigma$   $C_2$ . 7, A alone. Tension scale as for Plate 39, fig. 1.

FIG. 6.—As in fig. 5 later in the same experiment. The observations were as follows:—1,  $C_2$  alone. 2,  $C_1$  17.9  $\sigma$   $C_2$ . 3,  $C_1$  17.9  $\sigma$   $C_2$ . 4,  $C_1$  13.8  $\sigma$  A 4.1  $\sigma$   $C_2$ . 5, A 11.5  $\sigma$   $C_2$ . 6,  $C_1$  6.4  $\sigma$  A 11.5  $\sigma$   $C_2$ . Time 1 d.v. = 10  $\sigma$ . Tension scale as for Plate 39, fig. 1.

*Studies on the Flexor Reflex.—IV. After-Discharge.*

By J. C. ECCLES, M.A., D.Phil., Research Fellow of Exeter College, and  
Sir CHARLES SHERRINGTON, O.M., F.R.S., Physiological Laboratory, Oxford.

(Received January 3, 1931.)

[PLATES 41, 42.]

I. INTRODUCTION.

In many preparations the flexor reflex elicited by the application of a moderately strong break-shock to an ipsilateral afferent nerve has an after-discharge following the initial reflex discharge, even when the strength of the break-shock is such that it sets up no more than a single centripetal volley (Sherrington, 1921, *a*; 1921, *b*; Adrian and Forbes, 1922). The prolonged excitatory condition which must occur at some part of the central reflex pathway clearly has some affinity to the persistence of the c.e.s. which forms the basis of facilitation (Eccles and Sherrington, 1930). It is of interest therefore to investigate the effect of an antidromic volley on after-discharge. It must, however, be remembered that an antidromic volley set up during an after-discharge will be prevented from reaching some motoneurones by meeting centrifugal (reflex) impulses. Denny-Brown (1929, p. 273) observed that an antidromic volley set up during the after-discharge of either a flexor or extensor reflex (in response to a tetanic stimulation) was followed by a period of quiescence owing to a temporary lapse of the after-discharge. The duration of this period seemed to be too long for a central refractory period set up by the antidromic volley, so he suggested that there might be a temporary exhaustion of the central exciting agent (c.e.s.).

II. METHOD.

The general technique is as described previously (Eccles and Sherrington, 1931, *a*). In all cases the muscle (tibialis anticus) has been completely de-afferented. Tetanic stimuli have been provided by a neon-tube oscillator.

III. EXPERIMENTAL OBSERVATIONS.

1. *The Period of Quiescence.*

Plate 41, fig. 1, shows part of a series in which the antidromic volley was set up at various intervals after a single centripetal volley which was evoking a

reflex response accompanied by a considerable after-discharge (visible in both the electrical and mechanical records). In all the records where the antidromic volley was set up 15.8  $\sigma$  or more after the centripetal volley, the electrical record shows a period of *complete* quiescence following the motor action-current (this latter is a necessary consequence of setting up the antidromic volley in the intact motor nerve (*cf.* Eccles, 1931, p. 559)). After a quiescent period varying from 35  $\sigma$  to 60  $\sigma$ , a little after-discharge reappeared. The duration of this quiescent period is measured between the beginnings of the motor action-current and of the first action-current of the reappearing after-discharge; consequently it is about 5  $\sigma$  (allowing 2.5  $\sigma$  for the time of travel in the motor nerve fibres from the point of stimulation to the motoneurones) longer than the quiescent period existing in the motoneurones between the arrival of the antidromic volley and the recommencement of the after-discharge.

When the antidromic volley was set up 11.8  $\sigma$  or less after the centripetal volley, the period of quiescence was so short as to be almost unrecognisable, *e.g.*, in observation 6, Plate 41, fig. 1, where the stimulus-interval was 7.9  $\sigma$ . In those motor nerve fibres belonging to discharging motoneurones, the antidromic impulses and the initial centrifugal (reflex) impulses suffered a mutual blocking (*cf.* Eccles and Sherrington, 1931, *a*, p. 521), for the "modal" latent period was 11.4  $\sigma$ . The action-currents which occurred so soon after the motor action-current must have been due to early second discharges (*i.e.*, after-discharge) from some motoneurones (see p. 594). At this stimulus-interval it seems that the after-discharge was not appreciably affected by the antidromic volley\* (*cf.* observations 6 and 7, Plate 41, fig. 1). This forms the basis of one method of calculating the approximate tension developed by the after-discharge (see p. 588). In another experiment similar observations have been made. The period of quiescence was found to vary from 24.5  $\sigma$  to 60  $\sigma$ .

Plate 42, fig. 2, shows a series of observations from an experiment which had an after-discharge of much longer duration than either of the preceding experiments. The periods of quiescence in this series were 34  $\sigma$ , 47  $\sigma$ , and 25  $\sigma$  for the third, fourth and sixth observations respectively, but in a later observation of this experiment the after-discharge recommenced before the motor action-current subsided. However, in all the other (24) observations of this experiment the antidromic volley was followed by a recognisable period of quiescence of the after-discharge varying in duration from 18  $\sigma$  to 52  $\sigma$ . In

\* This is to be expected since the initial reflex impulses prevent the antidromic volley from reaching the motoneurones responsible for the after-discharge.



the exceptional case mentioned above the period of quiescence was probably as short as  $15\sigma$ .

The observations in these three experiments have been confirmed in four experiments where an antidromic volley was set up during the after-discharge following a reflex set up by repetitive stimulation. Fig. 3 shows an antidromic volley preventing all after-discharge for  $32\sigma$ . With the tetanic series the extreme values for the duration of this quiescent period were  $26\sigma$  and  $67\sigma$ .

In all the above experiments it has been observed that the weaker the after-discharge, the longer is the period of quiescence following an antidromic volley. For example the period of quiescence is the longer the later an antidromic volley is set up in any particular after-discharge.

## 2. *The Tension Developed by the After-discharge.*

In fig. 1 there is plotted for each stimulus-interval the maximum tension of the residual response left after subtracting the maximal motor response from

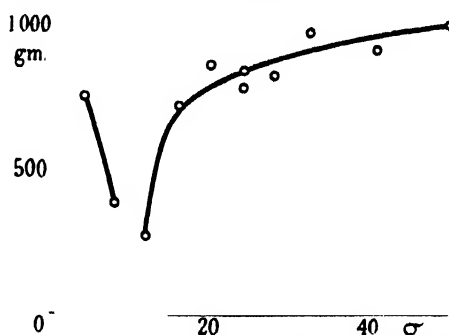


FIG. 1.—Tension of reflex response (ordinates) is plotted against the interval between the stimuli to the afferent and motor nerves (abscissae).

the total response,\* i.e., by the method of maximum tension differences (*cf.* fig. 2, Eccles, 1931). When the stimulus-interval was  $15.8\sigma$ , the motor twitch would follow the initial reflex response at such an interval that it would be maximal (the "modal" reflex-time was  $11.4\sigma$ ). The total response would, therefore, be compounded of the initial reflex response, the maximal motor

\* In the following discussion it will be assumed that, when elicited during the after-discharge, the motor twitch is maximal. This is not strictly correct for some of the motor units will be refractory owing to a preceding reflex discharge (the "line-busy" effect of Forbes, Whitaker, and Fulton, 1927; and of Gerard and Forbes, 1928). The subtraction of the maximal motor response from the total response really deducts both the motor response of the excitable units and the immediately preceding reflex response of the refractory units.

twitch, and a very small late after-discharge (observation 1, Plate 41, fig. 1). The value of 616 grm. left after subtraction of the motor twitch must be almost entirely due to the initial reflex response. Owing to the method of calculation (method of maximum tension differences (Eccles and Hoff, 1931)) this value would be about 1.1 times\* too large. If 560 grm. were the true value, 290 grm. of the reflex response would have to be accountable for as after-discharge, for 850 grm. was the maximal tension of the total reflex response.

The tension added by the after-discharge could also be calculated when the stimulus-interval was  $7.9 \sigma$ . It has been seen that there was then a maximal motor twitch followed by an apparently normal after-discharge. The tension, 328 grm., left after subtracting the motor twitch would, therefore, be about 1.1 times the tension of the after-discharge. This gives a value of just under 300 grm. and so there is good agreement with the previous calculation.

With intervals longer than  $15.8 \sigma$  there was a gradual increase in the tension of the residual response (fig. 1). This is an indication of an increase in the amount of after-discharge which was allowed through before the antidromic volley was set up. Even at  $50 \sigma$  this increase seems to be going on, and this corresponds with the electrical record which shows the action-currents of the after-discharge for at least this period. Similar observations were also made in experiment 19.5.30.

#### IV. DISCUSSION.

Our observations confirm those of Denny-Brown (1929, p. 273) in showing that a period of quiescence is produced by an antidromic volley set up during after-discharge. This period of quiescence cannot be due simply to the raised neurone-threshold (central refractory period) which follows the antidromic volley, for the neurone-threshold returns to normal in about  $10.5 \sigma$  (Eccles, 1931, p. 567). From experimental results the conclusion has been drawn that preformed c.e.s. is removed from a motoneurone by an antidromic volley (Eccles, 1931, p. 575), and this must be the basis of the long period of quiescence in the after-discharge. The commencement of fresh after-discharge following this period parallels the building up of fresh c.e.s. after the removal of preformed c.e.s. by an antidromic volley (Eccles, 1931, p. 579). The reappearance of the after-discharge would, therefore, be due to delayed excitatory impulses incident on the motoneurones after the antidromic volley.

As far as can be ascertained from the records, the quiescent period which follows an antidromic volley is always characterised by a *complete* cessation of

\* This unusually small ratio was indicated by some previous observations.

the after-discharge. The significance of this is apparent when it is remembered that the antidromic impulses are prevented from reaching some motoneurons by colliding with the centrifugal (reflex) impulses from these motoneurons. Since the time of travel to the spinal cord from the electrodes on the motor nerve is about  $2.5\sigma$ , such a collision occurs in the motor fibres of all those motoneurons which discharge an impulse during a period of  $5\sigma$  beginning  $2.5\sigma$  before and ending  $2.5\sigma$  after the setting up of the antidromic volley.\* If all the after-discharging motoneurons were responding asynchronously at only 20 per second, 10 per cent. of the motoneurons would be protected from the antidromic volley in this way, and in most records (*e.g.*, observations 1 and 2, Plate 41, fig. 1) their after-discharge should be easily recognisable if it continued throughout the period of quiescence.

The absolute character of the period of quiescence must therefore indicate that the motoneurons not subjected to the antidromic volley normally do not respond during this period. Since the period of quiescence occurs no matter where the antidromic volley occurs relative to the after-discharge, it must be concluded that *the rhythm of the after-discharge of any particular motoneurone is such that the interval between any two discharges is at least as long as the period of quiescence.*† Further, experiment shows that both the period of quiescence and the interval between successive discharges of a motoneurone are longer when observed later in the after-discharge than when observed earlier (*cf.* Eccles and Sherrington, 1930, Plate I, fig. 2). Thus experimental evidence indicates that the period of quiescence following an antidromic volley is probably related to the interval between the successive discharges of a motoneurone. It has been concluded that the period of quiescence following an antidromic volley is due to the removal of c.e.s. Can the interval between successive discharges of a motoneurone be caused by the disappearance of c.e.s. as a result of each discharge? Before considering this possibility it will

\* In addition there would be no antidromic impulses in those motor fibres which were refractory owing to the passage of a centrifugal (reflex) impulse just before the setting up of the antidromic volley.

† Since the after-discharge often does not regain its full strength immediately after the period of quiescence (*vide* observations 4 and 6, Plate 42, fig. 2), this period seems to last longer with some motoneurons than with others. Observations on the effect of an antidromic volley on the rhythmic responses of single motoneurons innervating soleus muscle show that an antidromic impulse usually prevents a reflex discharge for a period *longer* than the interval between two successive discharges at the normal rhythm of response (see p. 593). If the motoneurons innervating tibialis anticus muscle behave similarly, the first motoneurons to recover from the period of quiescence will be those which were not reached by the antidromic volley.

be well to examine an alternative explanation of the rhythm of reflex discharge, *e.g.*, after-discharge.

The description will be facilitated by reference to fig. 2 in which intensity of c.e.s. is plotted as ordinates against time as abscissæ. OT represents the

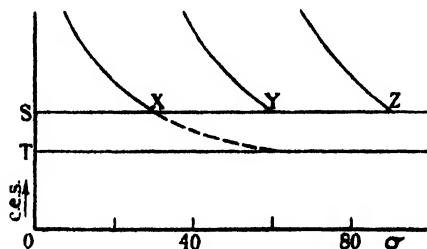


FIG. 2.

threshold value of c.e.s. for the resting motoneurone, and OS represents the supraliminal intensity of c.e.s. which is assumed to be present. The figure is drawn as if a reflex discharge had just occurred at S, so the threshold of the motoneurone is very high at first but gradually returns towards normal (OT) reaching the intensity OS at X. At this point a reflex discharge is again set up with the consequent raised threshold and subsequent recovery till another discharge is set up at Y, and so on for Z. Thus according to this hypothesis, the rhythm of discharge is dependent both on the curve of recovery of the neurone-threshold and on the intensity of the supraliminal excitement. Now it has been shown that the neurone-threshold returns to normal in about  $10.5 \sigma$  after an antidromic volley (Eccles, 1931, p. 567), and it has been argued that a similar recovery of neurone-threshold should follow a reflex-discharge (Eccles and Sherrington 1931, *b*, p. 552), an inference which is supported by a number of experimental observations. That being so the hypothesis just discussed cannot account for rhythms of discharge with a frequency less than 90 to 100 per second. Since the frequency of a discharge may be 15 per second or even lower (*cf.* Eccles and Sherrington, 1930, Plate 1, fig. 2), the above hypothesis seems inadequate to account for the experimental facts.

The hypothesis adverted to originally assumes that the preformed c.e.s. of a motoneurone is removed by a reflex discharge exactly as it is by an antidromic impulse, so c.e.s. has to be built up to neurone-threshold again before a second discharge can occur. The production of a rhythmic discharge of a motoneurone according to this hypothesis is shown schematically in fig. 3, where intensity of c.e.s. (ordinates) is plotted against time (abscissæ). OT represents threshold intensity of c.e.s. in a resting motoneurone. The figure

is drawn as if a reflex discharge had just occurred at T, and the recovery of the neurone-threshold is drawn so as to correspond approximately with that follow-

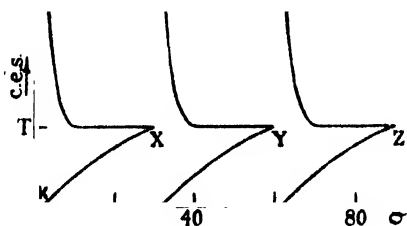


FIG. 3.

ing an antidromic volley. KX shows the progressive building up of c.e.s. (due to the incidence of delayed excitatory impulses) after the fall resulting from the discharge at T. When the intensity of c.e.s. reaches neurone-threshold at X a reflex discharge is immediately set up with a consequent raised neurone-threshold and lowered intensity of c.e.s. as at the beginning of the figure. A repetition of the cycle of events sets up a discharge at Y, and again at Z. According to this hypothesis a motoneurone is affected similarly by an antidromic volley and by a reflex discharge. That this is likely to be so is indicated by the following line of argument.

Anatomical considerations indicate that, after passing the axon hillock, the wave of excitability of the antidromic impulse traverses the surface of the cell body and the dendrites to their terminations. When a motoneurone discharges a centrifugal impulse, *i.e.*, a reflex discharge, this impulse must traverse the same path as that followed by the antidromic impulse; but, for the most part, the direction of travel would be in the reverse sense. If it be assumed that the reflex discharge is initiated at a synapse or the synapses on one dendrite, *e.g.*, at P in fig. 4, it is clear that, when it passes over the surface of the cell body, it will spread up the other dendrites in a way similar to an antidromic impulse. Therefore the effect on the synapses of these dendrites, and even on those synapses of the initially excited dendrite which did not give rise to the discharge, should be indistinguishable from the effect of the antidromic impulse\*—there should be a removal of the preformed c.e.s. It is also most probable that the synapse or synapses responsible for the reflex discharge would be similarly affected. Thus the available lines of evidence support the

\* In this connection it may be noted that the effects following a propagated disturbance in peripheral nerve are independent of the direction of travel.

hypothesis that a motoneurone is affected similarly by an antidromic volley and by a reflex discharge.\*

During a normal after-discharge the responses of individual motoneurones are out of phase with one another, hence the almost uninterrupted sequence of small action-currents in the electrical response. If an antidromic volley affected each motoneurone which it reached exactly as if a reflex discharge had taken place, the result would be really equivalent to a momentary synchronisation of the discharges of all these motoneurones (but see note \*). Those motoneurones which had discharged just previously would, of course, not be reached by the antidromic volley (*cf.* p. 590), so the condition of the motoneurones of the reflex "centre" would be as follows. Those which have discharged impulses less than  $5\sigma$  (or  $6\sigma$ , *vide* note \*, p. 590) before the antidromic volley is due to arrive at the "centre" are protected from the antidromic volley, but are affected by their own reflex-discharge as if an antidromic impulse had reached each at various times up to  $5\sigma$  or  $6\sigma$  before the antidromic volley itself arrives at the "centre" and affects all the other motoneurones. The duration of the period of quiescence is, therefore, only an approximate measure of the interval between successive discharges of those motoneurones responding with the highest frequency.

The shortest quiescent period which we have observed was about  $15\sigma$  in duration (measured from the beginning of the motor action-current to the beginning of the first action-current of the subsequent reflex discharge). This

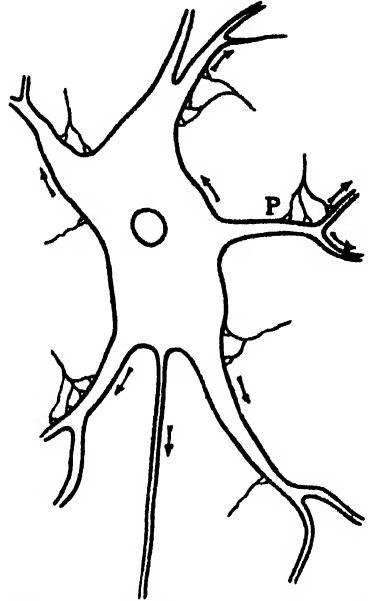


FIG. 4.—Diagram of the cell-body and parts of the dendrites and axon of a motoneurone showing a few terminal endings (*boutons*) of afferent fibres. The arrows on the surface indicate the probable direction of spread of an impulse (reflex discharge) set up at P.

\* Recent observations (Eccles and Hoff, 1931) show that during a rhythmic series of responses a motoneurone innervating soleus muscle is affected similarly by an antidromic impulse and by a reflex discharge. The earlier the antidromic impulse or the reflex discharge occurs in the rhythmic cycle the longer is the interval before the next reflex discharge.

corresponds to a frequency of nearly 70 per second, and so agrees well with the rhythm (85 to 60 per second) observed by Adrian and Bronk (1929) in one preparation. Usually the quiescent period was between  $20\sigma$  and  $50\sigma$  in duration, corresponding to rhythms of discharge of 50 and 20 per second respectively. Rhythms slower than 15 per second have not been observed.

The quiescent periods considered above followed an antidromic volley set up after the initial reflex discharge had passed (*vide* observation 1, Plate 41, fig. 1). If the antidromic volley is set up so that it collides with the initial reflex discharge, the quiescent period may be so brief as to be almost unrecognisable, *e.g.*, in observation 6, Plate 41, fig. 1, it is probably as short as  $10\sigma$ . In one experiment it was only  $6\sigma$  in duration. The antidromic volley does not, of course, reach the discharging motoneurones under these circumstances, but it serves to distinguish between the discharges of motoneurones which have already responded and the delayed initial discharges of other motoneurones (*cf.* Eccles and Sherrington, 1931, *a*, p. 522).

Thus in response to a single centripetal volley the initial reflex discharge of a motoneurone may be followed at an interval of  $6\sigma$  to  $10\sigma$  by a second discharge, but subsequent discharges occur at progressively longer intervals until they abruptly cease. The longest interval is usually between  $50\sigma$  and  $67\sigma$ . Somewhat similar results have been observed directly on the after-discharge of a single unit (Eccles and Sherrington, 1930, Plate 1, fig. 2). In this experiment the shortest interval found between successive responses was  $33\sigma$  and the longest  $196\sigma$ . The discrepancy between this latter value and the longest interval found by the present method may be due to the difference in the state of the spinal cord, for the present experiments were performed on acute spinal cats, the other on a chronic spinal dog.

So far it has been assumed that during after-discharge the motoneurones are being continually bombarded by excitatory impulses. The only possible explanation of this is the "delay paths" suggested by Forbes (1921, 1922). Some of the delay will be due to slow conducting fibres in peripheral nerve (*cf.* Erlanger and Gasser, 1930), some will be due to the conduction-time along complicated pathways in the spinal cord, and some to "synaptic" delay at internuncial neurones. The present conception of after-discharge, however, differs from that of Forbes, since it postulates that there is a gradual building up of c.e.s. by the successive arrival of delayed impulses before each reflex discharge is set up. As each response of the after-discharge occurs when the c.e.s. reaches neurone-threshold, the interval between any two discharges of a motoneurone is inversely proportional to the rate of increase of c.e.s. This

in turn is dependent both on the rate of incidence of the excitatory impulses, and on the rate of spontaneous disappearance of the c.e.s. It is to be noted that according to this conception of after-discharge it is impossible to have a c.e.s. of supraliminal intensity, for, as soon as threshold is reached, a discharge will be set up with the consequent removal of the c.e.s.

#### V. SUMMARY.

A period of quiescence follows an antidromic volley set up during the after-discharge of a reflex evoked either by a single centripetal volley or by a repetitive series of centripetal volleys (confirming Denny-Brown).

The duration of this period of quiescence (usually  $20\sigma$  to  $50\sigma$ ) is too long to be explained by the raised neurone-threshold (central refractory period) set up by the antidromic volley. It must be due to the removal of c.e.s. by the antidromic volley.

Since some motoneurones are protected from the antidromic volley by impulses which they have just discharged, the *complete* absence of after-discharge during the period of quiescence suggests that a motoneurone is affected by a reflex discharge in the same way as by an antidromic volley. This inference is supported by other experimental evidence and by an argument from general principles.

The conclusion arrived at is therefore that preformed c.e.s. of a motoneurone is removed by a reflex discharge, and c.e.s. has to be built up again by delayed excitatory impulses before another discharge can occur. Hence after-discharge depends on the continued arrival of delayed excitatory impulses, and not on a supraliminal c.e.s.

On the basis of these conceptions the duration of the period of quiescence should be approximately the same as the interval between successive discharges of the motoneurones.

One of us (J.C.E.) wishes to express his gratitude to the Medical Research Council for a personal grant, and we have to thank the Royal Society and the Rockefeller Foundation for grants towards the apparatus used in this research.

#### REFERENCES.

- Adrian, E. D., and Bronk, D. W. (1929). 'J. Physiol.,' vol. 67, p. 119.  
Adrian, E. D., and Forbes, A. (1922). 'J. Physiol.,' vol. 56, p. 301.  
Denny-Brown, D. E. (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 252.  
Eccles, J. C. (1931). 'Proc. Roy. Soc.,' B, vol. 107, p. 557.  
Eccles, J. C., and Hoff, H. E. (1931). "Unpublished Observations."



- Eccles, J. C., and Sherrington, C. S. (1930). 'J. Physiol.' vol. 60, p. 1.  
 Eccles, J. C., and Sherrington, C. S. (1931, a). 'Proc. Roy. Soc.,' B, vol. 107, p. 511.  
 Eccles, J. C., and Sherrington, C. S. (1931, b). 'Proc. Roy. Soc.,' B, vol. 107, p. 535.  
 Erlanger, J., and Gasser, H. S. (1930). 'Amer. J. Physiol.,' B, vol. 92, p. 43.  
 Forbes, A. (1921). 'Amer. J. Physiol.,' vol. 56, p. 273.  
 Forbes, A. (1922). 'Phys. Revs.,' vol. 2, p. 361.  
 Forbes, A., Whitaker, L. R., and Fulton, J. F. (1927). 'Amer. J. Physiol.,' vol. 82, p. 693.  
 Gerard, R. W., and Forbes, A. (1928). 'Amer. J. Physiol.,' vol. 86, p. 186.  
 Sherrington, C. S. (1921, a). 'Proc. Roy. Soc.,' B, vol. 92, p. 245.  
 Sherrington, C. S. (1921, b). 'Arch. Internat. Physiol.,' vol. 18, p. 620.

### EXPLANATION OF PLATES.

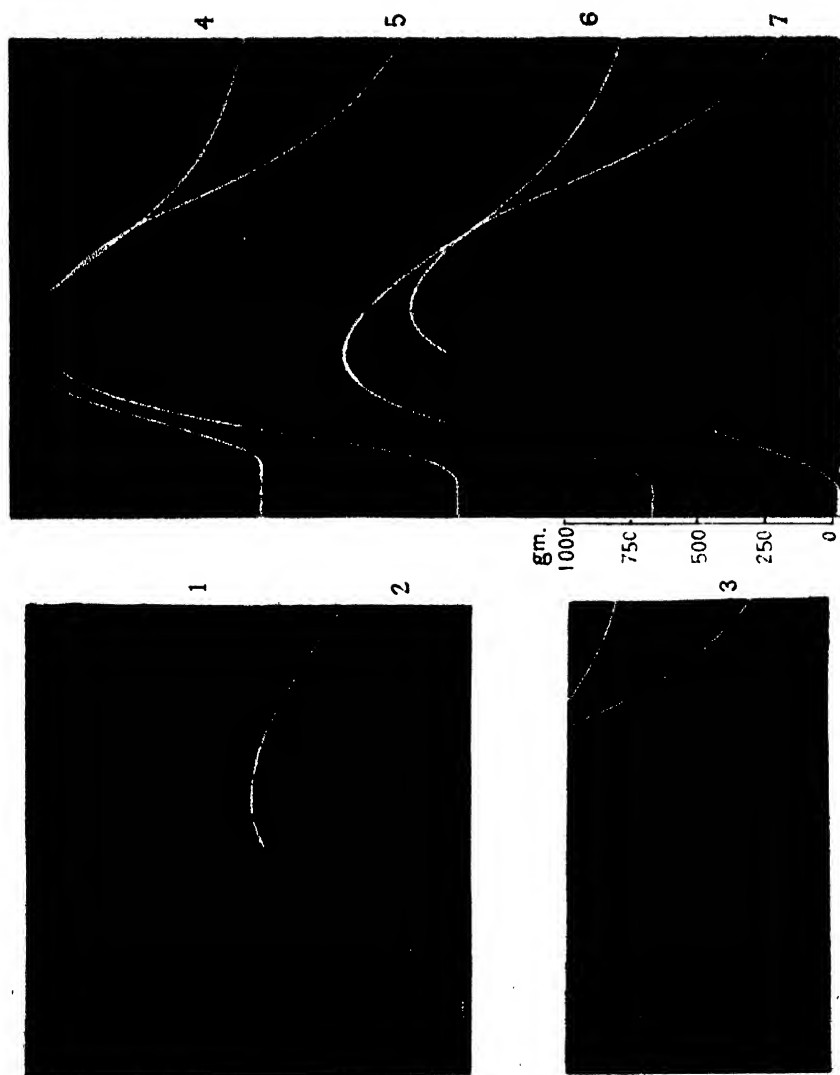
#### PLATE 41.

**FIG. 1.**—Electrical (dark lines) and mechanical (white lines) records of responses of tibialis anticus muscle to a single centripetal volley (C) set up in the posterior tibial nerve at various times relative to the application of a single stimulus (A) to the intact motor nerve (peroneal). The stimulus-intervals were as follows:—Observations 1, C 15·8σ A. 2, C alone. 3, C 28σ A. 4, A alone. 5, C 19·8σ A. 6, C 7·9σ A. 7, C 40·8σ A. Time, 1 d.v. = 10σ. Tension scale at side.

#### PLATE 42.

**FIG. 2.**—As in fig. 1, Plate 41, in another preparation. The observations are as follows:—1, C alone. 2, A alone. 3, C 45σ A. 4, C 197σ A. 5, A alone. 6, C 63σ A. The ends of the periods of quiescence are marked by the small white arrows. Tension scale as for Plate 41, fig. 1. Time, 1 d.v. = 10σ.

**FIG. 3.**—The observations are as follows:—1, A alone. 2, Reflex response to a repetitive series of centripetal volleys in posterior tibial nerve. 3, As in observation 2, but A is set up during the after-discharge. Time, 1 d.v. = 10σ. Tension scale at side.



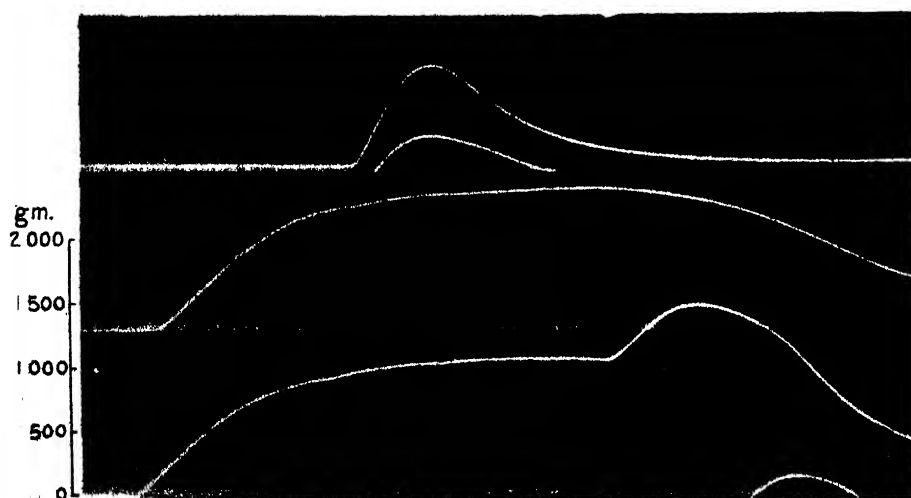
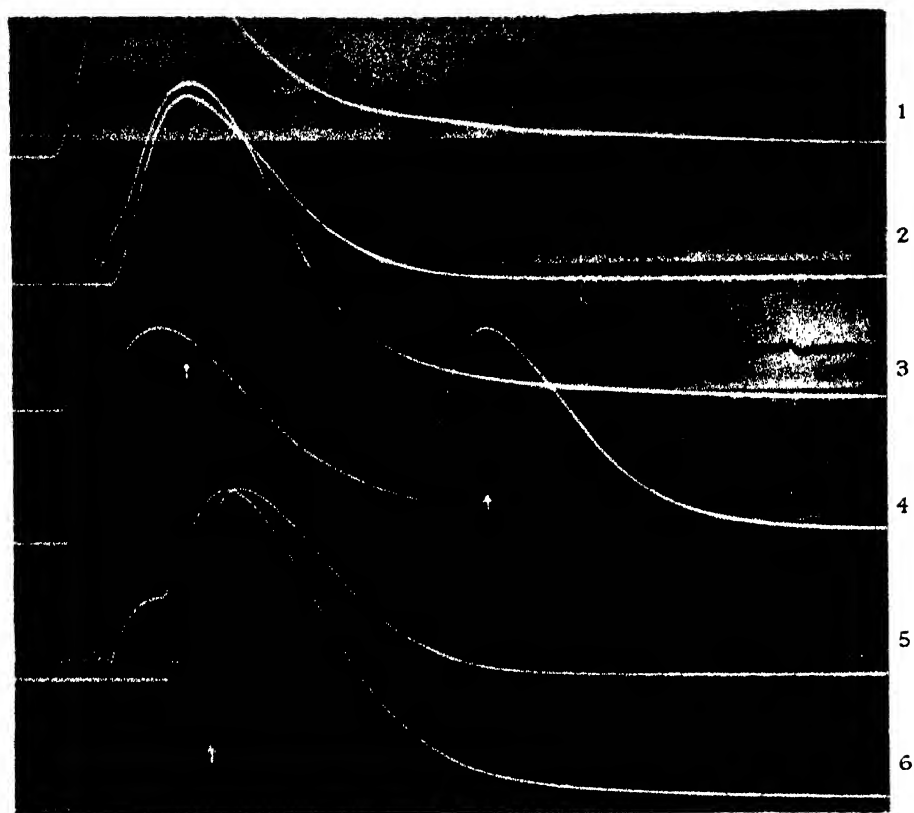


FIG. 3.

*Studies on the Flexor Reflex.—V. General Conclusions.*

By J. C. ECCLES, M.A., D.Phil., Research Fellow of Exeter College, and  
Sir CHARLES SHERRINGTON, O.M., F.R.S., Physiological Laboratory, Oxford.

(Received January 2, 1931.)

## I. INTRODUCTION.

The experiments described in the preceding four papers bear on various problems presented by reflex activity. Their results confirm some of the inferences already drawn elsewhere from other experimental work, and they allow certain further inferences. A brief prefatory statement of all these inferences and of the experimental evidence which allows them will advantageously introduce the description of the processes set up in the ipsilateral flexor centres of the spinal cord by a single centripetal volley and by a single antidromic volley. Then, finally, discussion of the theories of reflex excitation can be undertaken in the light of the present experimental observations. The statement treats of the subject in its present phase only; the references to relevant papers are therefore restricted in the main to the more recent ones.

## II. INFERENCES FROM EXPERIMENTAL OBSERVATIONS.

1. *The Convergence of Different Afferent Paths on the same Motoneurones*

The following evidence shows that this occurs:—

(a) *Histological*.—Each motoneurone receives its “*boutons terminaux*” from many individual afferent terminals (Cajal, 1903).

(b) *Physiological*.—Centripetal volleys set up in *different* afferent nerves excite the same motoneurones (Camis, 1909; Cooper, Denny-Brown, and Sherrington, 1926; 1927; Sherrington, 1929; Cooper and Denny-Brown, 1929; Eccles and Sherrington, 1930; 1931, *a*; 1931, *b*).

2. *The Central Excitatory State.*

This is the name given to an enduring excitatory condition set up by a centripetal volley in the reflex “centre.” The experimental evidence for its existence is as follows:—

(a) When separated by certain intervals (usually not longer than 20  $\sigma$ ), two centripetal volleys (either in the same or different afferent nerves) evoke

a reflex discharge from motoneurones which do not respond to either volley alone (Sherrington, 1929; Bremer, 1930, *a*; Eccles and Sherrington, 1930; 1931, *b*).

(*b*) When two centripetal volleys are separated by certain intervals (usually not longer than  $20\sigma$ ), the central reflex-time of the response to the second volley is greatly shortened (even to less than  $0.5\sigma$ ) (Eccles and Sherrington, 1931, *a*, p. 528).

Both these experiments show that an excitatory condition set up by the first centripetal volley in the reflex "centre" persists until the arrival of the second volley. On account of this property of existing for some time, this central excitatory condition is called the central excitatory state, c.e.s. The experimental observations (*a*) and (*b*) also show that for some motoneurones the c.e.s. produced by one centripetal volley sums with that produced by another volley.\* Since this happens under the most varied conditions, it can be concluded that in any motoneurone there is a summation of the c.e.s. produced by *individual* centripetal impulses which are separated by a sufficiently short interval. The duration of the c.e.s. set up by a centripetal volley is due partly to the temporal dispersion of the incident excitatory impulses (see section 5), and partly to the persistence of the c.e.s. produced by any particular excitatory impulse (Eccles, 1931, p. 580).

### 3. *Neurone-threshold.*

Since the size of two centripetal volleys can be chosen so that summation of the c.e.s. produced by both is necessary to set up a reflex discharge from motoneurones (Eccles and Sherrington, 1930), it follows that the c.e.s. produced by either volley is alone inadequate to evoke such a discharge. Therefore in order to set up a reflex discharge of a motoneurone the c.e.s. must reach a certain intensity. This intensity is called the *neurone-threshold* (*cf.* Goldscheider, 1898).

### 4. *Subliminal Fringe.*

When a centripetal volley is made large enough to evoke a reflex discharge from some motoneurones, *i.e.*, to produce a c.e.s. of threshold intensity in them, it also produces in other motoneurones a c.e.s. of subliminal intensity (Denny-Brown and Sherrington, 1928; Eccles and Sherrington, 1931, *b*, p. 539). These latter motoneurones are said to be in the subliminal fringe produced by that centripetal volley.

\* The argument in the case of (*b*) depends on sections 3 and 5 below.

5. *Temporal Dispersion of Centripetal Impulses incident on a Motoneurone as the Result of a Single Centripetal Volley.*

The following experimental observations are difficult to interpret in any other way :—

(a) The central reflex-time of the response to a centripetal volley is greatly shortened if another volley precedes it by certain intervals. When the volleys are in different afferent nerves, the central reflex-time usually reaches a minimum when the interval between them is  $6\sigma$  to  $8\sigma$  (Eccles and Sherrington, 1931, *a*, p. 527).

(b) The central reflex-time is shortened when the centripetal volley is increased in size (Eccles and Sherrington, 1931, *c*, p. 517).

(c) There is a temporal dispersion of the reflex discharge evoked by a centripetal volley (Forbes and Gregg, 1915; Eccles and Sherrington, 1931, *a*).

(d) A centripetal volley evokes a reflex discharge after an antidromic volley which latter reaches the motoneurones *after* the foremost impulses of the centripetal volley. In order to set up such a reflex discharge some excitatory impulses must have been incident on the motoneurones *after* the antidromic volley (Eccles, 1931, p. 569).

(e) When a centripetal volley reaches the reflex "centre" immediately after an antidromic volley, it evokes a reflex response with a central reflex-time longer than normal (Eccles, 1931, p. 565).

(f) When an antidromic volley reaches the reflex "centre" after the foremost impulses of a centripetal volley, very little or no c.e.s. can be detected for several sigmata, but in some cases this period is followed by a considerable increase in the c.e.s. (Eccles, 1931, p. 579).

(g) An antidromic volley set up during an after-discharge produces a complete cessation of all after-discharge for a period varying from  $15\sigma$  to  $60\sigma$  (Denny-Brown, 1929, p. 273; Eccles and Sherrington, 1931, *c*, p. 586).

6. *Refractory Period following an Antidromic Volley.*

(a) After an antidromic impulse reaches a motoneurone, the neurone-threshold is raised so that for a period of  $5\sigma$  to  $6\sigma$  no reflex discharge can be set up. The neurone threshold does not fall to normal for a further period of about  $5\sigma$  (Eccles, 1931, p. 567).

(b) The period of raised threshold following an antidromic volley is not altered by a preceding antidromic volley except in so far as the second volley is delayed owing to the refractory period following the first volley (Eccles, 1931).

(c) Two antidromic volleys can be set up at such a short interval apart that the second reaches the reflex "centre" only  $2.4\sigma$  after the first. The absolutely refractory period following an antidromic volley must be less than this (Eccles, 1931, p. 568).

*7. Refractory Period following a Reflex Discharge (cf. Bremer, 1930, b).*

After a centripetal volley evokes a reflex discharge from a motoneurone there is a period during which the neurone-threshold is raised. In some motoneurons this period is less than  $16\sigma$ , and probably is identical in duration with the refractory period following an antidromic volley (Eccles and Sherrington, 1931, b, p. 553). When the period is longer than  $30\sigma$  it can be shown that its long duration is due, not to a refractory period, but to an inhibition set up by inhibitory impulses in the centripetal volley (Eccles and Sherrington, 1931, b, p. 550). In those cases where the duration of raised threshold lies between  $16\sigma$  and  $30\sigma$  experimental evidence does not distinguish between inhibition and refractory period as causes of this duration of the unresponsiveness (Eccles and Sherrington, 1931, b, p. 551).

*8. An Antidromic Impulse removes Preformed c.e.s. from a Motoneurone which it reaches.*

An antidromic volley reaching the reflex "centre" between two centripetal volleys greatly reduces the facilitation of the second volley by the first. This is not due to the raised neurone-threshold (refractory period) produced by the antidromic volley (Eccles, 1931, p. 575).

*9. When a Reflex Discharge is set up in a Motoneurone, there is a Removal of Preformed c.e.s.*

(a) An antidromic volley set up during an after-discharge produces a complete cessation of all after-discharge for a period varying from  $15\sigma$  to  $60\sigma$ . This duration is too long to be explained as a refractory period set up by the antidromic volley (Denny-Brown, 1929, p. 273; Eccles and Sherrington, 1931, c, p. 589). Since some motoneurons are protected from the antidromic volley by impulses which they have just discharged, the complete absence of after-discharge during the period of quiescence suggests that a motoneurone is affected in a similar way by a reflex discharge and by an antidromic volley.

(b) An antidromic impulse reaching a rhythmically discharging motoneurone affects that motoneurone in the same way as a reflex discharge (Eccles and Hoff, 1931).

### III. CENTRAL PROCESSES SET UP BY A SINGLE CENTRIPETAL VOLLEY OR BY A SINGLE ANTIDROMIC VOLLEY.

On the basis of the inferences which have just been considered it is now possible to sketch in the outlines of some of the processes set up in the ipsilateral flexor centres of the spinal cord both by a single centripetal volley and by a single antidromic volley.

#### 1. *The Central Effects of a Centripetal Volley (provided that it contains no Inhibitory Impulses).*

According to the intensity of the effects which they produce it is possible to divide centripetal volleys into three classes.

(a) *A Centripetal Volley which does not give rise to a Reflex Discharge.*—The excitatory impulses incident on a motoneurone have a considerable temporal dispersion, and each produces its quantum of c.e.s. so that the maximum intensity of c.e.s. is usually reached in about  $6\sigma$  to  $8\sigma$  (cf. fig. 6, Eccles and Sherrington, 1931, a). Thereafter the intensity of c.e.s. declines until a zero value is reached about  $20\sigma$  after the initial rise. The maximum intensity of c.e.s. varies in different motoneurones according to the number of incident excitatory impulses, but in none does it reach neurone-threshold.

(b) *A Centripetal Volley which evokes a Reflex Twitch.*—As in the previous instance the excitatory impulses incident on a motoneurone have a considerable temporal dispersion, but now the intensity of the c.e.s. of some motoneurones reaches threshold with the result that a reflex discharge is instantly set up, i.e., an impulse passes down the axon (motor nerve fibre) of each of these motoneurones. The interval between the incidence of the foremost impulses of the centripetal volley and the setting up of the reflex discharge may be as short as  $2.5\sigma$  in some motoneurones or as long as  $6\sigma$  to  $8\sigma$  in others; hence there is often a considerable temporal dispersion of the individual impulses of the reflex discharge.

The reflex discharge of any motoneurone is accompanied by a reduction in the intensity of its c.e.s., and by an absolutely refractory period lasting not longer than  $2.4\sigma$ . The neurone-threshold does not, however, reach a normal value for about another  $8\sigma$  (duration of relatively refractory period). Excitatory impulses incident on motoneurones after the reflex discharge build up c.e.s., but not to a threshold intensity for the response is a reflex twitch (cf. Eccles and Sherrington, 1931, a, p. 522).

Besides thus evoking a reflex discharge from some motoneurones the centripetal volley also gives rise to a subliminal c.e.s. in other motoneurones (the



subliminal fringe). The condition in these is identical with that considered in the previous section.

(c) *A Centripetal Volley which evokes a Repetitive Discharge (after-discharge) from some Motoneurones.*—When the centripetal volley is still larger than that employed in evoking a reflex twitch, the initial reflex discharge of many motoneurones is set up as in the previous section, but excitatory impulses incident on some motoneurones after this initial discharge are sufficient to raise the c.e.s. to threshold again and so set up a second discharge. In some motoneurones this may be repeated several times. Following a large centripetal volley there may be a bombardment of some motoneurones by excitatory impulses for as long as  $50\sigma$  or even  $500\sigma$ . Only some motoneurones of the reflex centre will respond repetitively; others will respond only once as in the previous section; others will be in the subliminal fringe.

## 2. *The Central Effects of an Antidromic Volley.*

An antidromic impulse reaching any motoneurone removes preformed c.e.s., and is followed by an absolutely refractory period lasting for not longer than about  $2.4\sigma$ . A single centripetal volley, however, cannot set up a reflex discharge until about  $5\sigma$  after the antidromic volley has reached the motoneurone. The additional interval is due to the time consumed in building up the c.e.s. to threshold value. Neurone-threshold does not fall to a normal value until about  $10.5\sigma$  after an antidromic volley (end of the relatively refractory period).

## IV. THEORETICAL DISCUSSION.

From the considerations on p. 597 it is clear that the central excitatory state is a convenient term for expressing the experimental facts of summation of the central excitatory conditions set up by centripetal volleys. Since it is now generally agreed that this summation occurs (*cf.* Fulton, 1926, p. 350; Adrian and Bronk, 1929, p. 146; Sherrington, 1929, p. 343; Bremer, 1930, *a*; Eccles and Sherrington, 1930; Forbes, Davis and Lambert, 1930, p. 165), the theoretical discussion need only be concerned with ideas on the nature and location of the central excitatory state.

The ideas expressed in the papers quoted above show a considerable difference of opinion between different observers. Thus Fulton (1926, p. 350) favours the view that each excitatory nerve impulse produces a quantum of exciting agent, a chemical substance, which sums with other quanta formed at the same or neighbouring points by other impulses. This summation is pictured as centering about the axon hillock (p. 359). Forbes, Davis and Lambert

(1930), on the other hand, suggest that the electrical responses of successive nerve impulses summate by a process allied to that observed in the "retention of action-current" of crustacean nerve (Levin, 1927), or the negative after-potential of vertebrate nerve (Amberson and Downing, 1929; Gasser and Erlanger, 1930). The position of Adrian and Bronk (1929) is not so clearly defined. They suggest that there may be two different mechanisms involved in the spinal flexor reflex. (a) Direct transmission of centripetal impulses to motoneurons by a process not differing greatly from that involved in the conduction of an impulse from one section of a nerve fibre to the next. (b) A more lasting excitatory state (? chemical substance) produced in the synaptic region as a result of the passage of each impulse. Bremer (1930, a) emphasises the undoubted similarity existing between summation in the reflex arc (*addition latente centrale*) and in the partly curarised nerve muscle preparation (*addition latente périphérique*). Sherrington (1925, 1929) and Eccles and Sherrington (1930, p. 25) are non-committal with regard both to the nature and the location of the c.e.s.

The removal of the c.e.s. of a motoneurone by an antidromic impulse (Eccles, 1931) indicates that c.e.s. is restricted to those parts of the motoneurone accessible to such an impulse. It is generally agreed that a nerve impulse in peripheral nerve traverses the surface membrane of the axis cylinder, so it may be assumed that an antidromic impulse also traverses the surface membrane of the motoneurone and its dendrites. It is therefore likely that c.e.s. is confined to this surface membrane. It does not seem possible that a chemical substance, such as is postulated by Fulton, would be restricted to the surface membrane of the motoneurone, or, further, that it would be removed by an antidromic impulse or a reflex discharge.

The hypothesis of Forbes, Davis, and Lambert (1930) is also difficult to reconcile with the effect of an antidromic impulse in removing c.e.s. If c.e.s. resembled negative after-potential, one would expect that an antidromic impulse would add to that already existing. Moreover negative after-potential seems to be an abnormal condition present in excised nerve, for immediately after excision of a nerve it is comparatively small, but then it proceeds to increase in extent and duration. Also it is closely related to the supernormal phase (Gasser and Erlanger, 1930) which itself is an abnormal condition of nerve (Adrian, 1920). For these reasons it does not seem likely that central summation depends on a process allied to the negative after-potential of peripheral nerve.

There is, however, another process in peripheral nerve which may give a

clue to the nature of the c.e.s., namely the "local excitatory state" (Lucas, 1910, 1912). It is an excitatory process localised at the stimulated region of the excitable tissue, and it is capable of summation. Though very short in duration in peripheral nerve (about  $1\sigma$  at most), it is longer in other excitable tissues, *e.g.*, it has a duration of at least  $8\sigma$  in the heart. Moreover, the removal of local excitatory state by a nerve impulse is analogous to the removal of c.e.s. by an antidromic volley, and, again, the disappearance of local excitatory state resulting from the setting up of a propagated disturbance (nerve impulse) is analogous to the disappearance of c.e.s. resulting from the setting up of a reflex discharge.

Thus it seems likely the c.e.s. is a specialised manifestation of the local excitatory state (*cf.* Sherrington, 1921). According to the membrane theory (Bernstein, 1912; Lillie, 1922), the latter is a partial depolarisation of the polarised membrane surrounding the axis cylinders of nerve fibres, so on analogy c.e.s. is probably a depolarisation of those parts of the surface membranes of motoneurons on which the excitatory impulses impinge, *i.e.*, the synaptic membranes.

This hypothesis as it stands does not explain the well established fact that there is a summation of the c.e.s. produced by excitatory impulses reaching different synapses of the same motoneurone. In addition to this summation the "synaptic" delay of the response to a centripetal volley can be almost reduced to vanishing point by a suitably timed centripetal volley in *another* afferent nerve (Eccles and Sherrington, 1931, *a*, p. 527). In order to reconcile the hypothesis with these facts it must be assumed that the changes produced at any synapse are not restricted on the surface membrane of the motoneurone to the actual locus of that synapse, but that the changes affect at least the immediately adjacent synapses. The profusion of "*boutons terminaux*" (Cajal, 1909, fig. 110, p. 311) belonging to the endings of a single afferent fibre on a motoneurone would serve to give opportunity for immediately adjacent synapses (*boutons*) to be excited by impulses in any two afferent fibres having endings on the same motoneurone.

The parallel which Adrian and Bronk (1929, p. 145) draw between the discharges of receptor organs and of motoneurons, and the similarity which Bremer (1930, *a*) draws attention to between *addition latente centrale* and *addition latente périphérique* are both in harmony with the above hypothesis, and it is to be noted that it attempts to explain the facts of excitation in the central nervous system by assuming no properties which are unknown in peripheral nerve (*cf.* Lucas, 1917, p. 2).

V. SUMMARY.

A brief statement is made of those inferences drawn from experimental observations, which are related to the problems dealt with in the preceding four papers.

Then follows a short description of the processes set up in the ipsilateral flexor centres by a single centripetal volley and by a single antidromic volley.

In the light of the existing data there is a discussion of various hypotheses bearing on the nature of central excitation in reflex activity.

One of us (J. C. E.) wishes to express his gratitude to the Medical Research Council for a personal grant.

REFERENCES.

- Adrian, E. D. (1920). 'J. Physiol.,' vol. 54, p. 1.  
 Adrian, E. D., and Bronk, D. W. (1929). 'J. Physiol.,' vol. 67, p. 119.  
 Amberson, W. R., and Downing, A. C. (1929). 'J. Physiol.,' vol. 68, p. 19.  
 Bernstein, J. (1912). "Electrobiologie." Braunschweig, vi.  
 Bremer, F. (1930, a). 'C. R. Soc. Biol.,' vol. 103, p. 509.  
 Bremer, F. (1930, b). 'C. R. Soc. Biol.,' vol. 103, p. 513.  
 Cajal, Ramon y (1903). "Trab. Lab. Investigaciones Biolog.," Madrid, vol. 2, p. 129.  
 Cajal, Ramon y (1909). "Histologie Systeme Nerveux," Paris.  
 Carnis, M. (1909). 'J. Physiol.,' vol. 39, p. 228.  
 Cooper, S., and Denny-Brown, D. E. (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 365.  
 Cooper, S., Denny-Brown, D. E., and Sherrington, C. S. (1926). 'Proc. Roy. Soc.,' B, vol. 100, p. 448.  
 Cooper, S., Denny-Brown, D. E., and Sherrington, C. S. (1927). 'Proc. Roy. Soc.,' B, vol. 101, p. 262.  
 Denny-Brown, D. E. (1929). 'Proc. Roy. Soc.,' B, vol. 104, p. 252.  
 Denny-Brown, D. E., and Sherrington, C. S. (1928). 'J. Physiol.,' vol. 66, p. 175.  
 Eccles, J. C. (1931). 'Proc. Roy. Soc.,' B, vol. 107, p. 557.  
 Eccles, J. C., and Hoff, H. E. (1931). *Unpublished observations.*  
 Eccles, J. C., and Sherrington, C. S. (1930). 'J. Physiol.,' vol. 69, p. 1.  
 Eccles, J. C., and Sherrington, C. S. (1931, a). 'Proc. Roy. Soc.,' B, vol. 107, p. 511.  
 Eccles, J. C., and Sherrington, C. S. (1931, b). 'Proc. Roy. Soc.,' B, vol. 107, p. 535.  
 Eccles, J. C., and Sherrington, C. S. (1931, c). 'Proc. Roy. Soc.,' B, vol. 107, p. 586.  
 Forbes, A., Davis, H., and Lambert, E. (1930). 'Amer. J. Physiol.,' vol. 95, p. 142.  
 Forbes, A., and Gregg, A. (1915). 'Amer. J. Physiol.,' vol. 37, p. 118.  
 Fulton, J. F. (1926). "Muscular Contraction and Reflex Control of Movement," Baltimore.  
 Gasser, H. S., and Erlanger, J. (1930). 'Amer. J. Physiol.,' vol. 94, p. 247.  
 Goldscheider, A. (1898). "Die Bedeutung der Reize in Lichte der Neuronlehre," Leipzig.  
 Levin, A. (1927). 'J. Physiol.,' vol. 63, p. 113.  
 Lillie, R. S. (1922). 'Phys. Rev.,' vol. 2, p. 1.  
 Lucas, K. (1910). 'J. Physiol.,' vol. 39, p. 461.  
 Lucas, K. (1912). 'Proc. Roy. Soc.,' B, vol. 85, p. 495.  
 Lucas, K. (1917). "Conduction of the Nervous Impulse," London.  
 Sherrington, C. S. (1921). 'Arch. Internat. Physiol.,' vol. 18, p. 620.  
 Sherrington, C. S. (1925). 'Proc. Roy. Soc.,' B, vol. 97, p. 519.  
 Sherrington, C. S. (1929). 'Proc. Roy. Soc.,' B, vol. 105, p. 332.

*The Osmotic Changes in Some Marine Animals.*

By R. MARGARIA, Turin.

(From the Marine Biological Laboratory, Plymouth.)

(Communicated by A. V. Hill, F.R.S.—Received December 11, 1930.)

Since Bottazzi's (1897) first determinations of the osmotic pressure of the body fluids of various marine animals many researches have been performed by other authors, particularly in reference to the permeability of the membranes separating the body from its surroundings. Bottazzi (1897, 1906, 1908, *b*) investigated individuals belonging to very different groups of animals, and found that the osmotic pressure of the body fluids of marine invertebrates, and of elasmobranchs, is very similar to that of the surroundings, while the osmotic pressure of the blood of teleosts is quite different.

Changing the osmotic pressure of the medium, the osmotic pressure of most marine invertebrates, and of elasmobranchs, was shown to change in the same direction (L. Fredericq, 1882, 1904; Quinton, 1897; Dakin, 1908) and to reach, finally, the value of the former. The blood of teleosts is much more independent of the medium, for it was shown to change only about 30 per cent. in concentration, on transferring the animals from sea water to fresh water or *vice versa* (Dakin, 1908; Dekhuyzen, 1904; Sumner, 1905); other authors, however (Fredericq, 1904; Garrey, 1905) could not find even these variations.

Chemical analyses showed that the blood of invertebrates has a saline composition very similar to that of the external medium, while the composition of the blood of elasmobranchs has a salinity only about one-half that of the surroundings, the deficit in osmotic pressure being made up by organic substances, particularly urea. The salinity of the blood of teleosts is only about 2/5 of that of sea water, this ratio almost corresponding to that of the depressions of the freezing points.

There are a few marine species of invertebrates which can regulate the osmotic pressure of their body fluid to a value different from that of the medium. This has been demonstrated for example in some worms (*Nereis diversicolor*, Schlieper,

1929; *Gunda ulva*, see Weil and Pantin, 1931), and in the shore crab, *Carcinus maenas*, by Duval (1925) and by Schlieper (1929). L. Fredericq had observed before (1904) that such crabs, after three days in diluted sea water ( $\Delta = 1.19^\circ$ ) had an osmotic pressure of their blood corresponding to  $\Delta = 1.68^\circ$ , i.e., sensibly higher than the surroundings; while Duval (1925) noted that the concentration of the blood after 15 days in diluted sea water was still higher than that of the medium, and the same as it was after 24 hours in the same water. Schlieper, moreover, showed that the blood of shore crabs, which had presumably always lived in a diluted medium, i.e., in the water of the Bay of Kiel ( $\Delta = 0.90^\circ$ ), had a  $\Delta$  of  $1.5^\circ$ .

Evidently the steady difference maintained between the internal and the external fluids is a function (i) of the permeability of the membranes, or organs, separating the two fluids, particularly of the gills, and (ii) possibly of the activity of the secretory organs in eliminating water (or salts) contained in excess in the blood. It seems very unlikely that the maintenance of this difference is principally due to the excretory function of the kidneys or alimentary tract, the gills being largely permeable to water and salts; for then we should be forced to admit that these organs are continually at work in the teleosts, in the elasmobranchs and in the homoiosmotic invertebrates, to remove large quantities of salts from their blood. Though such excretory activity may occur, the action of the inlet membranes is almost certainly more important.

Dakin suggests (1912) that, at least partially, the difference of concentration is due to the presence in the internal fluid of certain sodium salts, to whose anions, as to the undissociated salts, the membrane is impermeable, being permeable, however, to all other ions and salts. This peculiar impermeability would be characteristic of the living membrane only; in the case of plaice eggs, death of the cell causes diffusion of NaCl till the same osmotic concentration is reached.

Another mechanism proposed by Dakin, to explain the difference of concentration between the internal and the external media, involves a molecular affinity between the colloid contents of the internal fluid, and the ions present in it (Moore, Roaf and Webster, 1911). Dakin finds in this mechanism a reason for the maintenance of a certain salinity in the plasma of animals passing from sea water to fresh water. It is certain, however, that so great a difference of concentration as sometimes exists between the two sides of a living membrane is not simply due to such a physico-chemical equilibrium as Dakin supposes, but rather, as we shall see later, to an active living process,

by which the physico-chemical equilibrium is never reached, a process requiring energy and accompanied by oxygen consumption.

An example of a sensible difference of concentration between the two sides of a living membrane is that described by Straub (1929) and confirmed by A. V. Hill (1930, *b*), between the yolk and the white of a hen's egg. The difference of osmotic pressure is very remarkable, being about two atmospheres ; it is probably due to an active living process, though not necessarily aerobic.

The intervention of active energetic processes in the regulation of the osmotic pressure of the internal fluid of aquatic organisms may be exhibited by following the variations of concentration in the internal fluid of animals put into a medium of altered osmotic concentration. If no living process occurred to stop the passage of dissolved constituents, or water, through the membrane, the variation of the osmotic concentration of the internal fluid might be expected to be nearly a logarithmic function of the time, complicated perhaps by changes (*a*) in the permeability of the membranes separating the internal from the external fluid, occurring when the concentration of these changed ; and (*b*) in the circulation rate. The present investigation is concerned with the way in which such variations of the osmotic pressure of the blood of certain marine animals occur ; it deals with teleosts, elasmobranchs and invertebrates, kept in diluted sea water at various concentrations, for different lengths of time.

#### METHOD.

The experiments were performed at Plymouth, from July to September, 1930. The animals were taken from the common tank of the aquarium, the chemical composition and osmotic pressure of the sea water therein being known, and put in small baths or jars, in the same tank water, diluted with tap water ; the Plymouth tapwater may be considered for this purpose as distilled water.

Various species of crabs, dogfish and congers were used. The blood was collected in small glass tubes, which were then closed with rubber stoppers to prevent evaporation. From crabs it was obtained by cutting off a leg, external fluid from the gills being prevented from mixing with the blood by holding the animal in a dry towel ; each crab was used only for a single collection of blood. From dogfish it was obtained by cutting off the head. From congers it was collected directly from the branchial artery, after destroying the brain.

The osmotic pressure was determined by means of the thermal method of measuring vapour pressures devised by A. V. Hill (1930, *a*), with the same

technique as used for human blood (Margaria, 1930) ; it differed from the latter only in the facts : (a) that the blood was not equilibrated with a  $\text{CO}_2$  gas-mixture ; and (b) that the solution against which the vapour pressure of the blood was determined varied, consisting either of the medium in which the animals lived, or of sea water diluted to such an extent as to bring its osmotic pressure near to that of the fluid to be examined, in order to make the readings, and therefore also the errors concerned, the smallest possible.

Calibration of the thermopiles was carried out by observing the deflection of the galvanometer caused by putting on one face filtered outside sea water diluted to 1/5, on the other face tap water. The vapour pressure of the undiluted sea water, which is very constant in composition, was determined against a known NaCl solution, and found to be equal to that of a solution containing 3.41 g. of NaCl per 100 g. of water. The tank water was somewhat more concentrated owing to evaporation.

Over the range of concentrations involved in these observations the depression of vapour pressure and hence the osmotic pressure are fairly exactly proportional to the concentration, expressed in grammes of salt in 100 g. of water\*—see Hill's paper (1930, a). *The osmotic pressure, therefore, will be expressed in terms of the isotonic solution of NaCl, in grammes per 100 g. of water, without other designation.*

## RESULTS.

(A) The experiments on *Maia squinado* and on *Portunus depurator* are summarised in Tables I and II and figs. 1 and 2. They show that the osmotic pressure of the blood of these animals comes rather soon to nearly the same value as that of the external fluid ; the final point is reached a little sooner by *Portunus depurator* than by *Maia squinado*, which is presumably due to the smaller size of the former. Experiments on the same kind of animal and in the same dilution, performed on different days, show different velocities of dilution of the blood ; this is probably due to the daily variations of room temperature, the velocity being greater the higher the temperature.

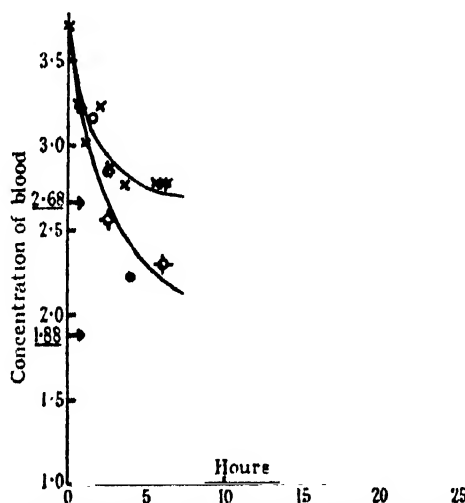
\* The depression of freezing point ( $\Delta$ ) is not so accurately proportional to the concentration, and the osmotic pressure at  $20^\circ \text{C}$ ., therefore, not so exactly proportional to the  $\Delta$ . Approximately the  $\Delta$  of a solution of  $n$  g. of NaCl in 100 g. of  $\text{H}_2\text{O}$  is  $0.585 n^\circ$ . The osmotic pressure of such a solution at  $T^\circ$  absolute is  $0.0255 nT$  atmospheres, or  $7.47 n$  atmospheres at  $20^\circ \text{C}$ .



Table I.—Osmotic Pressure (o.p.) of the Blood of *Maia squinado* (fig. 1).

ster (T.W.) (o.p. = 3.75). Blood: 3.80, 3.87, 3.91, mean 3.86.			
(A) In 10/14 tankwater (o.p. = 2.68)	Aug. 5	After 2h. 30m.	2.74, 2.93, 2.
	" 5	" 6h.	2.81, 2.72.
	" 30	" 30m.	3.86, 3.12.
	" 30	" 1h.	3.01.
	" 30	" 2h.	3.13.
	" 30	" 3h. 30m.	2.77.
	" 30	" 5h. 30m.	2.78.
	" 30	" 10h. 10m.	2.67.
	" 31	" 24h.	2.64.
(B) In 1/2 tank water (o.p. = 1.88)	Aug. 5	After 2h. 30m.	2.48, 2.65.
	" 5	" 6h.	2.43, 2.16.
	Sept. 4*	" 45m.	3.14, 3.29.
	" 4	" 1h. 30m.	3.16, 3.16.
	" 4	" 2h. 30m.	2.84.
	" 4	" 4h.	2.12.

\* Of this lot of crabs one died after 1 hour, three others died between 3 and 4 hours after the beginning of the experiment.

FIG. 1.—Change of concentration of the blood of *Maia squinado* put in diluted media.

Ordinate = concentration of blood expressed in grammes of NaCl per 100 g. of water.

Abscissa = time from the beginning of the experiment.

In 10/14 T.W. (o.p. = 2.68) × Experiment begun August 30.

\* Experiment of August 5.

In 1/2 T.W. (o.p. = 1.88) ○ Experiment of September 4.

⊙ Experiment of August 5.

**Table II.—Osmotic Pressure of the Blood of *Portunus depurator* (fig. 2).**

Blood was obtained from several crabs and mixed; the number of crabs used for each observation is given in brackets.

(A) Difference of concentration between blood and medium, in various media	Aug. 6 " 7 " 7 " 7	After $\infty$ h. in 3.75 " 25h. in 3.13 " 25h. in 2.81 " 25h. in 1.87	0.0774 (3). 0.0143 (3). 0.0150 (3). 0.0539 (3).
(B) In 2/3 tank water (o.p. = 2.50)	Aug. 7 " 7 " 7	After 30m. " 1h. 45m. " 4h. 15m.	3.23 (3). 2.76 (2). 2.60 (3).
(C) In 2/3 tank water (o.p. = 2.41)	Aug. 26 " 26	After 4h. 30m. " 7h. 20m.	2.50 (3). 2.53 (3).
(D) In 2/3 tank water (o.p. = 2.43)	Aug. 18 " 18 " 18 " 19	After 30m. " 1h. 30m. " 3h. 30m. " 21h.	3.16 (3). 2.77 (3). 2.67 (3). 2.49 (3).
(E) After 24 hours in 2/3 tank water, crabs were put in tank water again (o.p. = 3.65)	Aug. 19 " 19 " 19 " 19	After 30m. " 1h. 30m. " 4h. 15m. "	2.95 (2). 3.37 (2). 3.61 (2). 3.65 (2).
(F) In 1/2 tank water (o.p. = 1.88)	Aug. 7 " 7 " 7	After 30m. " 1h. 45m. " 4h.	2.89 (2). 2.53 (2). All died.

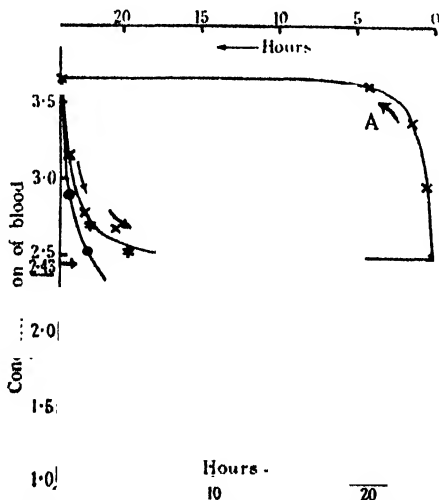


FIG. 2.—Change of concentration of the blood of *Portunus depurator* put in diluted media and later replaced in its original medium. Details as in fig. 1. The time scale at the top applies to the returning curve A.

In 2/3 T.W. (o.p. = 2.43)  $\times$  Experiment of August 19.

### Experiment of August 7.

✕ Experiment of August 26.

In 1/2 T.W. (o.p. = 1.88)

### Experiment of August 7.

The dilution of the medium to 0.6 tank water is considered to be about the limit of the dilution of the medium, since in some experiments most of the crabs in tank water diluted to one-half died after a few hours.

The rapid fall of the curve to the level of the medium suggests that the membrane separating the internal from the external fluid is osmotically quite permeable; and since these crabs do not show sensible lasting variations in weight under such conditions, as was demonstrated by Schlieper (1929) and by Hukuda (1931), the membrane must be completely permeable to water and salts.

The condition of free osmotic equilibration between the inside and the outside of these animals is shown also by the shape of the curves of dilution of the blood, which are almost logarithmic. If the logarithm of the difference between the actual and the final value be plotted as a function of the time, the result is either a straight line or a curve with slight convexity towards the abscissa. If the gill membrane had a constant osmotic permeability, and if no changes occurred in the body during dilution, one would expect the curves so plotted to be straight lines; in the living animals, however, owing to alterations of the membrane in the course of the dilution of the internal fluid, and particularly to variations of the circulatory conditions, the simple relation is not exactly obtained; the later changes are always relatively less rapid than the earlier ones.

The curve of concentration of *Portunus depurator* acclimatised to dilute sea water and then put in undiluted sea water again, reproduces the characteristics of the curve of dilution; the similarity between the two curves obtained in opposite directions confirms the passive behaviour of this crab in the determination of the osmotic pressure of its internal fluid.

A few experiments were made also on lobsters and on *Echinus esculentus*: these animals show the same osmotic behaviour as *Maia squinado* and *Portunus depurator* differing only in the time-scale of the changes involved, owing to differences in size and circulatory activity.

(B) It is known, since the observations of Duval and Schlieper, that *Carcinus maenas* can maintain a difference of concentration between its own internal medium and the external one. In my experiments the capacity of these crabs to regulate the osmotic pressure of their blood has been confirmed. In fact (1) the curve of dilution of the blood of *Carcinus maenas* far from being logarithmic, is a straight line till (2) a constant value is reached which, for media of osmotic pressure = 1.88 or 1.10, is sensibly higher than that of the medium; (3) the velocity of this process of dilution is much less for this crab

than for the others referred to above, though its size is much less than that of *Maia squinado* and only just greater than that of *Portunus depurator*; and (4) the curves of concentration of the blood of animals acclimatised to media of low osmotic pressure and then put back in normal sea water are quite different from those found for dilution.

Table III.—Osmotic Pressure of the Blood of *Carcinus maenas* (fig. 3).

(A) Animals from tank water (o.p. = 3.65)	Aug. 11	—	3.58, 3.62, 3.59, 3.56; mean 3.59.
Animals from tank water (o.p. = 3.75)	July 31	—	3.74, 3.79; mean 3.76.
(B) In 1/2 tank water (o.p. = 1.83)	Aug. 11	After 1h. 30m.	3.39, 3.37.
	" 11	" 4h.	3.20, 3.18.
	" 11	" 8h.	2.95, 2.62.
	" 12	" 26h. 30m.	2.59, 2.61.
	" 13	" 48h.	2.61, 2.69.
	" 14	" 77h.	2.66.
	" 16	" 120h.	2.72, 2.72.
(C) In 1/3 tank water (o.p. = 1.21)	Aug. 28	After 4h. 30m.	2.83.
	" 28	" 10h.	2.19.
	" 29	" 23h. 45m.	2.09.
	" 31	" 70h.	2.00.*
	Sept. 1	" 94h.	2.17.*
	" 2	" 120h.	2.14.*
(D) In 1/4 tank water (o.p. = 0.94)	July 31	After 1h.	3.65.
	" 31	" 2h. 30m.	3.26.
	" 31	" 5h.	2.67.
(E) In 1/4 tank water (o.p. = 0.91)	Aug. 11	After 1h. 30m.	3.35.
	" 11	" 4h.	2.97.
	" 11	" 8h.	2.47.
	" 12	" 26h. 30m.	2.21.
	" 14	" 77h.	1.91, 1.81
(F) After 5 days in 1/2 tank water the crabs were put in tank water again (o.p. = 3.62)	Aug. 25	After 1h. 20m.	3.00.
	" 25	" 3h.	3.08.
	" 25	" 5h.	3.13.
	" 25	" 8h. 10m.	3.18.
(G) After 5 days in 1/2 tank water the crabs were put in tank water again (o.p. = 3.67)	Sept. 1	After 2h. 50m.	2.77.
	" 1	" 6h.	3.04.
	" 1	" 14h.	3.42, 3.49, 3.42, 3.44.
	" 2	" 24h. 30m.	3.56.
(H) After 5 days in 1/3 tank water the crabs were put in tank water again (o.p. = 3.67)	Sept. 1	After 5h.	3.08.
	" 1	" 10h. 30m.	3.18.
	" 2	" 24h. 10m.	3.40.

\* These crabs stayed 36 hours in 1/2 tank water before being put in 1/3 tank water.

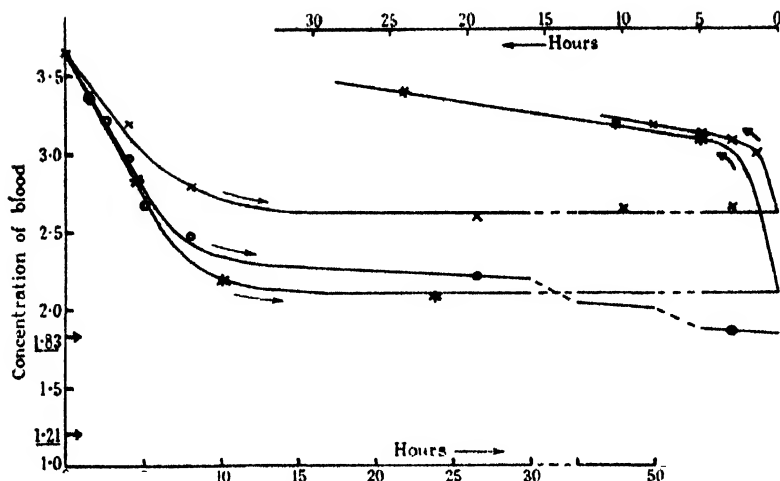


FIG. 3.—Change of concentration of the blood of *Carcinus maenas* put in diluted media. Details as in figs. 1 and 2.

In 1/2 T.W. (o.p. = 1.83) × Experiments begun August 11 and August 25.

In 1/3 T.W. (o.p. = 1.21) \* Experiment begun August 28.

In 1/4 T.W. (o.p. = 0.92) ○ Experiments begun July 31 and August 11.

Though from the experiments of Schlieper it appears that the osmotic pressure of the medium can be brought as low as 0.7, in my experiments, with a dilution to 0.94, a steady state was not reached. The osmotic pressure of the blood decreased slowly, but continuously, even after more than three days; those crabs caught at Plymouth, at least, are not able to live permanently in water diluted to such an extent. The results obtained by Schlieper may be due to the fact that he did not leave the crabs a sufficiently long time in the dilute water to ensure the attainment of the steady state. Some of these crabs in my experiments could stay a relatively long time in distilled water (Plymouth tap water); they died only after 8 to 12 hours, showing by that time a considerable degree of swelling. The present observations agree with those of Duval (1925), from whose experiments it appears that at a concentration of 1.08 ( $\Delta = 0.63^\circ$ ) *Carcinus maenas* cannot live indefinitely.

Since these crabs, as is known from the observations of Schlieper (1929) and of Hukuda (1931), swell only to a small extent and for a short time after being put in a diluted medium, we must conclude from the facts above discussed, i.e., from the peculiarities of the curves of fig. 3, that their membranes are permeable to water and to salt, but that such permeability is under the regulation of a living active process.

(C) *Portunus puber*, although zoologically closely related to *Portunus*

*depurator*, does not live as the latter does, exclusively in deeper water (5 to 55 m.), namely, where the medium is very constant, but is usually found rather near the coast, and for this reason it is subjected to the variations of concentration of the sea water which occur in estuaries during the tides. In this respect its conditions of life are more similar to those of *Carcinus maenas*, though the latter lives still nearer the coast and may undergo still greater

Table IV.—Osmotic Pressure of the Blood of *Portunus puber* (fig. 4).

(A) Difference of o.p. between the crabs' blood and the tank water in which they lived 0.023, 0.066.

(B) In 2/3 tank water (o.p. = 2.42)	Aug. 8	After 30m.	3.31, 3.23.
	" 8	" 2h. 20m.	3.06, 3.00.
	" 8	" 4h. 20m.	2.81, 2.90.
	" 8	" 7h. 20m.	2.71, 2.61.
	" 9	" 22h. 50m.	2.39.
(C) In 2/3 tank water (o.p. = 2.44)	Sept. 1	After 3h.	3.02.
	" 1	" 6h.	3.04.
	" 1	" 14h.	2.61, 2.54.
(D) In 1/2 tank water (o.p. = 1.83)	Sept. 1	After 30m.	3.41, 3.18.
	" 1	" 1h. 30m.	2.93.
	" 1	" 3h.	2.85.
	" 1	" 7h.	2.55.
	" 1	" 10h.	2.29.
	" 2	" 25h.	2.01.

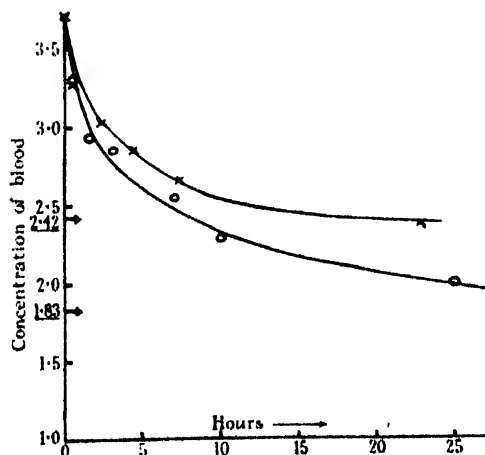


FIG. 4.—Change of concentration of the blood of *Portunus puber* put in diluted media. Details as in fig. 1.

In 2/3 T.W. (o.p. = 2.42) × Experiment begun August 28.

In 1/2 T.W. (o.p. = 1.83) ○ Experiment begun September 1.

variations in the concentration of its surrounding medium. From these considerations one would expect the capacity to regulate the osmotic pressure of the internal fluid in *Portunus puber* to lie between that of *Carcinus maenas* and that of *Portunus depurator*.

The curves in fig. 4 show that this really is the case. For (1) the rate of dilution of the blood is very slow, a constant value of the osmotic pressure being reached only after more than 20 hours, for dilution of the medium to  $\frac{2}{3}$  tank water, and after more than 30 hours, for dilution to  $\frac{1}{2}$  tank water; (2) the shape of the curve is not a logarithmic one, as can be seen if the logarithm of the difference between the actual and the final value be plotted against time: such curves resemble much more those obtained with *Carcinus maenas* than with other crabs.

Differing from *Carcinus maenas* the blood concentration of *Portunus puber* goes to a final value equal to that of the medium, so that this crab is not able to maintain indefinitely a difference of concentration between the internal and the external fluids; it can only restrain the rate of variation of concentration. A dilution of the medium to 0.5 T.W. seems to be the limit which this crab can stand.

These crabs, from the observations of Hukuda, do not swell appreciably when placed in a dilute medium. Their membranes therefore are permeable to water and salts, though permeation may be restrained, but not stopped, by an active living process.

(D) The experiments performed on various species of dog-fish (*Scoyllium*) were quite similar to those on *Portunus depurator* and on *Maia squinado*; the concentration of the blood reached that of the external fluid very soon (5 to 6 hours) according to a nearly logarithmic process. These animals, however, have a low resistance to dilution, the limit of osmotic pressure being 2.8; at 2.6 (75 per cent. sea water) animals previously acclimatised to 3.0 and apparently in good condition, as well as others taken directly from tank water, died within a few hours.

Another striking difference between these animals and the crabs named above is their behaviour in swelling. Hukuda found that dog-fish put in diluted sea water swell to an amount corresponding to the dilution, and that such swelling is permanent; this would explain their low resistance to dilution of the medium.

These facts, together with the other observation that the chemical composition of the blood of elasmobranchs is so different from that of sea water, would lead to the conclusion that the membranes of elasmobranchs are quite im-

Table V.—Osmotic Pressure of the Blood of Dog-fish (*Scyllium*) (fig. 5).

(A) Difference of o.p. between the fish's blood and the tank water in which it lived : 0.024, 0.002, -0.027.

B) In 4/5 tank water (o.p. = 2.92)*	Aug. 20	After 2h.	3.35.
	„ 20	„ 6h.	3.01.
	„ 21	„ 24h.	2.96.
	„ 21	„ 30h.	3.05.
	„ 22	„ 48h.	3.01.
C) In 3/4 tank water (o.p. = 2.72)	Aug. 28	After 1h.	3.11, 3.04.
	„ 28	„ 3h. 15m.	2.87, 2.81.
	„ 28	„ 6h.	2.80.
	„ 28	„ 10h. 20m.	2.81.
	„ 29	„ 24h.	Died.
D) In 3/4 tank water (o.p. = 2.75)	Aug. 25	After 30m.	3.40, 3.37.
	25	„ 1h. 30m.	3.20, 3.16.
	25	„ 4h. 30m.	3.08, 2.89.

\* Large animals and of various sizes were used.

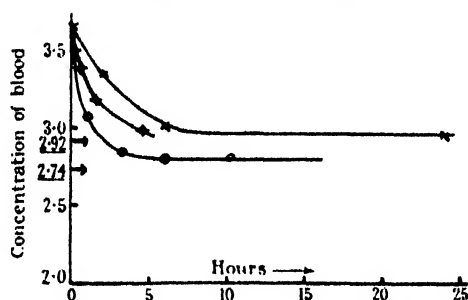


FIG. 5.—(Change of concentration of the blood of dog-fish (*Scyllium*) put in diluted media. Details as in fig. 1.

In 4/5 T.W. (o.p. = 2.92)      × Experiment begun August 20.  
 In 3/4 T.W. (o.p. = 2.72)      ○ Experiment begun August 28.  
 In 3/4 T.W. (o.p. = 2.75)      ·× Experiment of August 25.

permeable to salts and other dissolved substances, but completely permeable to water. The phenomenon of permanent swelling indicates the incapacity of the kidneys to eliminate water and dissolved substances present in excess in the organism. The urine of these animals was demonstrated by Bottazzi (1906) to have, under normal conditions, the same osmotic pressure as blood; it would seem, from these experiments, that not only the osmotic pressure but also the rate of secretion may be a constant character of the urine. It would be interesting to make further experiments on the activity of the kidneys under such conditions, and on the changes of weight of animals immersed in media



more concentrated than the normal, instead of more dilute. The latter test would prove definitely whether the membranes are in fact ideally semi-permeable, or whether the swelling observed by Hukuda is merely a symptom of abnormality in the animals, only casually corresponding to the dilution of the blood.

(E) The experiments upon congers (*Conger vulgaris*) show that the concentration of the blood falls, with dilution of the medium, very slowly till the concentration of the latter reaches about 0.3 (about 10 per cent. sea water); below this the concentration of the blood falls more rapidly till a stage is reached at which the animals die. The minimum concentration of the environment in which life is still possible appears for these animals to be just about 0.3. Why they die at this concentration of the medium it is not possible from the present observations to say. In solutions more dilute than 0.3 a great quantity of mucus becomes clearly visible as a precipitate on the surface of the animal, and passes into the surrounding water, which soon becomes dirty. The importance of the mucus in the resistance of similar animals (fresh water eels) to changes of concentration of the medium was emphasised a long time ago by P. Bert (Régnaud, 1891) and more recently by

Table VI.—Osmotic Pressure of Blood of Conger (*Conger vulgaris*) (fig. 6).

(A) Animals living in tank water (o.p. = 3.65): 1.31, 1.29.

(B) After 24 hours in diluted medium—

Concentration of medium....	2.55	1.21	0.365.
Concentration of blood.....	1.23	1.19	1.08, 1.08.

(C) August 25.—After 24 hours in 7/10 tank water (o.p. = 2.55), then 72 hours in 1/2 tank water (o.p. = 1.82): 1.24.

(D) August 28.—After 24 hours in 1/10 tank water (o.p. = 0.365), then in tap water: 1.16. The animal swelled up and was dying.

(E) September 2.—After 36 hours in 1/20 tank water (o.p. = 0.183): 0.885. The animal swelled up and was dying. Large hæmorrhages in the caudal part of the body; large production of mucus.

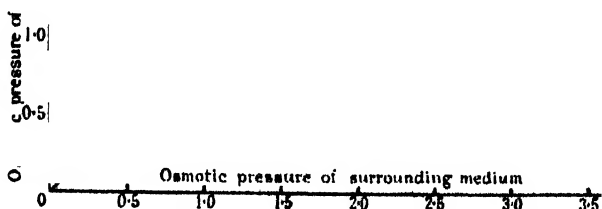


FIG. 6.—Osmotic pressure of the blood of *Conger vulgaris*, as a function of that of the external medium.

Duval (1925); the latter showed that such animals, deprived mechanically of the mucus by rubbing, swell when put in more concentrated media, and the osmotic pressure of the blood shows much greater variations than in normal animals.

It may be supposed that this precipitation of the mucus in media less concentrated than 0.3 is not a sign of the bad conditions of the conger but the cause of it; the mucus when precipitated probably loses its protecting properties. The precipitation might be due to the low concentration of the salts, or of some specific ion, in the surrounding medium.

It is interesting to point out that Duval (1925), in the fresh water eel subjected to concentration of the medium, found the same characteristic changes in the osmotic pressure of the blood as are here described for the conger, but reversed; which means that the mechanism by which animals can keep an osmotic pressure of the blood different from that of the surroundings is the same for animals living in a more concentrated as for those living in a more dilute medium.

The observations of Hukuda show that congers in a diluted medium swell, but osmotic pressure and swelling have not been determined at the same time so that we cannot say if the swelling is proportional to the dilution of the blood, as was demonstrated for dog-fish.

From the behaviour of the osmotic pressure of the blood in normal and in dilute media we can conclude that the gill membranes of congers are almost impermeable to water and salts, and only at great dilutions become slightly permeable to water.

#### DISCUSSION.

How can such great differences of osmotic pressure between the internal and the external fluids be maintained in some marine animals? It seems inconceivable that, the gill membranes being permeable to salts and water, the regulation of the osmotic pressure is maintained only by the kidneys, especially in view of the fact that, as appears from Bottazzi's experiments, the capacity of the kidneys of these animals to form concentrated or dilute urine is very small. It is much more likely that the regulation of the osmotic pressure of the internal fluid of such animals is maintained by the gills.

The impermeability in *Carcinus maenas* (and to some degree also in *Portunus puber*) is certainly not a passive property of the membrane, which appears to be easily and immediately permeable to water and salts when the external concentration is altered; an active living process must be involved.

It is not possible to say whether, in the elasmobranchs, the impermeability

to salts and urea is a property of the membrane, behaving as an ideal semi-permeable membrane, or not. The facts (i) that the curve of dilution of the blood when the animals are put in a diluted medium is so regular, and (ii) that the swelling is permanent and proportional to the dilution, would permit the hypothesis that the chemical composition of the blood of elasmobranchs is not kept up by an active process, but is due to a physical peculiarity of the membrane. The hypothesis, however, of a membrane passively impermeable to a substance so diffusible as urea, is not supported by a knowledge of any other membrane, animal or not, completely impermeable to that substance but at the same time permeable to water. Further observations on the osmotic pressure, as well as on the chemical composition of the blood and urine of these animals, and on their swelling when put in diluted or concentrated media, may throw light on this question.

A slight dilution of the blood of teleosts occurs in media more dilute than sea water but more concentrated than the blood itself. Without data on the swelling of these animals, when put in dilute media, we cannot say if the dilution of the blood under such conditions is due to taking up water or to eliminating dissolved substances from the body. The gill membrane in congers, therefore, being osmotically permeable, the difference of concentration between the internal and the external media must be maintained by an active living process. This conclusion is supported by the shape of the curve of the osmotic pressure of the blood as a function of the osmotic pressure of the medium; in fact we could not conceive any mechanism of simple diffusion by which a decrease of the osmotic pressure of the medium to a value *higher* than that of the internal fluid might cause a *decrease* of the osmotic pressure of the internal fluid itself. (See also Duval (1925) for similar experiments on the fresh water eel.) It is necessary to admit the intervention of a living process of some kind.

The mechanism by which this difference of concentration is maintained between the internal and the external fluids has recently been discussed by A. V. Hill (1930, b, 1931). A difference of concentration on the two sides of a living membrane is not a new phenomenon in physiology, since many fluids secreted or excreted have a different osmotic pressure from the internal fluids, which implies osmotic work in the cells. Such work, however, which till a few years ago seemed to be a peculiarity of certain complex organs, was demonstrated by Straub (1929) in the membrane of a single cell, the yolk of the hen's egg; and an analogous effect could be reproduced *in vitro* by the same author (1930, a) simply by a difference of electric potential maintained between the two sides of a cellophane membrane separating two salt solutions. Straub,

therefore, suggests as a possibility that a difference of electrical potential between the two sides of a membrane may be a cause of the steady difference of concentration which exists between them. More recently (1930, *b*) he has given a purely chemical model showing a similar steady difference.

The maintenance of such a difference of concentration implies a continual expenditure of energy, in reversing osmotic effects occurring spontaneously. This was supposed by Straub, in the case of the hen's egg, to involve oxygen consumption; a comparison, however, by Hill (1930, *b*) of the rate of decrease of the difference of osmotic pressure between yolk and white, for eggs kept in air and in oxygen-free hydrogen respectively, shows that oxygen consumption is at any rate not a necessity for the process. Even, however, if the energy required for maintaining osmotic differences in the case of the hen's egg need not be derived from an oxidative process, it is nevertheless very likely to be in organisms like those concerned in these experiments. When oxygen is available we might expect that the oxygen consumption of animals, while maintaining a difference of osmotic pressure between their body fluids and their surroundings, would be greater. The difference of oxygen consumption, however, due to such maintenance, might be very small; it has been calculated by Straub (1929), for the hen's egg, where the difference of osmotic pressure between yolk and white is about two atmospheres, to be equivalent to about 0.01 cal. per day, or only 0.036 per cent. of the total metabolism as calculated by Štěpánek (1904): so that, even assuming a very low efficiency, the increase of oxygen consumption due to such osmotic work might not be detected by the usual methods available for these animals.

An increase of 22 per cent. in oxygen consumption in *Carcinus maenas* in diluted media has been pointed out by Schlieper (1929); similar results have been claimed in a worm, *Nereis diversicolor*, by Tarussov (1927) and by Schlieper (1929), and in other marine animals by Raffy and Fontaine (1930). The increase in oxygen consumption appeared to be greater with greater differences of osmotic pressure between the internal and external fluids. Such increases, however, of oxygen consumption, which were interpreted by Schlieper as due to the increase of the osmotic work required, present very great experimental variations and are far too large to be attributed simply to an increase of osmotic work. The irregularity of the data so obtained makes it very probable that such increases in oxygen consumption must be attributed, at least in part, to enhanced activity of the animals. Shore crabs, placed in tap water at Plymouth, after 5 hours were so vivacious, though swollen, that I trusted they could stand overnight, which they did not. The

vivacity of these animals, contrary to what happens in other crabs, was evidently a symptom of abnormal conditions.

*Summary.*

1. The osmotic pressure of the blood of *Maia squinado* and of *Portunus depurator* has been tested ; it is very near the value of the medium in which the animals live, generally a little higher. These crabs cannot survive in a medium more dilute than about half the concentration of sea water ; the osmotic pressure of their blood, when they are put in dilute media, falls rapidly along an approximately logarithmic curve to the level of the medium. It is deduced that the gill membrane is permeable to water and salts, and that these animals are not able to resist osmotic equilibration between the internal and the external fluids.

2. The osmotic pressure of the blood of *Carcinus maenas* has the same value as that of the sea water in which it lives ; this crab, however, can survive a very long time in much more dilute media than those mentioned above, namely in sea water diluted to  $1/3$ . The osmotic pressure of its blood under such conditions decreases, but not to the level of the external medium, the crab being able to maintain a difference of osmotic pressure of about 0.9 (expressed in grammes of NaCl in 100 g. of water) between the internal and the external fluids. From this fact, together with the curve of dilution of its blood and the absence of swelling, it is deduced that the gill membrane of this animal is permeable to water and salts, but that the animal is able to regulate osmotically the concentration of its blood.

3. The blood of *Portunus puber* has the same osmotic pressure as the medium in which it lives. The minimum concentration compatible with life is about half that of sea water. From the curve of dilution of the blood when the animal is put in a dilute medium it is argued that although this crab is not able to maintain permanently a difference of concentration between the internal and external fluids, it can supply a certain osmotic resistance. This peculiarity is related to the conditions of life of these animals, which are intermediate between those of *Maia squinado* and *Portunus depurator* on the one hand, and those of *Carcinus maenas* on the other.

4. Elasmobranchs (*Scyllium*) cannot maintain a difference of concentration between the internal and the external fluids, and the limit of dilution of the medium is that of sea water diluted to  $3/4$ . The curve of dilution of their blood is approximately logarithmic, which is interpreted as evidence of the free permeability of the gills to water. From the chemical composition of the

blood of these animals, together with their behaviour in swelling, it is deduced that the gill membrane behaves as though it were ideally semi-permeable.

5. The osmotic pressure of the blood of teleosts (*Conger vulgaris*) is very different from that of the medium in which they live, being about equal to that of 1.3 g. of NaCl in 100 g. of water; it diminishes, but not greatly, with dilution of the medium; for a dilution of sea water to 1/10, the osmotic pressure of the blood decreases by 17 per cent. From the curve, however, of dilution of the blood, it is deduced that the body is osmotically permeable, and that the difference of concentration between the internal and the external fluids is maintained by an active process. The limit of dilution of the medium for these animals is to about 1/10 sea water; the lowering, below a certain limit, either of the concentration of the total salts, or of some specific ion, affects either the mucus or the cells on the surface of the animal.

6. The mechanism of osmotic regulation is discussed.

I am much indebted to Professor A. V. Hill for advice, suggestions and help during the course of this work; to Miss M. Hetherington and to Dr. K. Hukuda for considerable assistance in the experiments; and to Dr. E. J. Allen, Dr. C. M. Yonge, and all the staff of the Plymouth Laboratory for their hospitality and kindness.

#### REFERENCES.

- Bert, P. (1871). 'C.R. Acad. Sci.,' vol. 73, pp. 382, 464.  
 Bottazzi, F. (1897). 'Arch. ital. Biol.,' vol. 27, p. 61.  
 Bottazzi, F. (1906). 'Arch. Fisiol.,' vol. 3, pp. 416, 495, 547.  
 Bottazzi, F. (1908, a). 'Arch. Fisiol.,' vol. 5, p. 243.  
 Bottazzi, F. (1908, b). 'Ergeb. Physiol.,' vol. 7, p. 161.  
 Dakin, W. J. (1908, a). 'Biochem. J.,' vol. 3, p. 258.  
 Dakin, W. J. (1908, b). 'Biochem. J.,' vol. 3, p. 473.  
 Dakin, W. J. (1912). 'Intern. Rev. Hydrobiol. Hydrogr.,' vol. 5, p. 53.  
 Dekhuyzen, C. (1904). 'Proc. Roy. Acad. Amsterdam,' vol. 7.  
 Duval, M. (1925). 'Ann. Inst. Oceanog. Monaco,' N.S., vol. 2, p. 233.  
 Fredericq, L. (1882). 'Bull. Acad. Roy. Belg.,' vol. 4, p. 209.  
 Fredericq, L. (1884). 'Arch. Zool., exp. gen.,' vol. 3, p. 34.  
 Fredericq, L. (1904). 'Arch. Biol.,' vol. 20, p. 709.  
 Garrey, W. E. (1905). 'Biol. Bull.,' vol. 8, p. 257.  
 Hill, A. V. (1930, a). 'Proc. Roy. Soc., A,' vol. 127, p. 9.  
 Hill, A. V. (1930, b). 'Trans. Faraday Soc.,' vol. 26, p. 667.  
 Hill, A. V. (1931). "Eldridge Johnson Lectures, Philadelphia" (*in the press*).  
 Hukuda, K. (1931) (*in preparation*).  
 Margaria, R. (1930). 'J. Physiol.,' vol. 70, p. 417.  
 Moore, Roaf, H. E., and Webster, E. (1911). 'Biochem. J.,' vol. 6, p. 110.  
 Pantin, C. F. A. (1931). 'Journ. expt. Biol.,' vol. 8, p. 82.

- Quinton, R. (1897). 'C. R. Soc. Biol., Paris,' vol. **39**, p. 935.
- Raffy, A., and Fontaine, M. (1930). 'C. R. Soc. Biol., Paris,' vol. **104**, p. 466.
- Régnard, P. (1891). "Recherches expérimentales sur les conditions physiques de la vie dans les eaux." Masson, Paris.
- Schlieper, C. (1929). 'Z. verg. Physiol.,' vol. **9**, p. 478.
- Schlieper, C. (1930). 'Biol. Rev.,' vol. **5**, p. 309.
- Štěpánek, O. (1904). 'Zbl. Physiol.,' vol. **18**, p. 188.
- Straub, J. (1930, a). 'Trans. Faraday Soc.,' vol. **26**, p. 674.
- Straub, J. (1930, b). 'Chem. Weekblad.,' vol. **27**, No. 50.
- Straub, J., and Hoogerduyn, M. J. J. (1929). 'Rev. Trav. chim. Pays-Bas,' vol. **48**, p. 49.
- Sumner, F. B. (1905). 'Bull. U.S. Bureau Fisheries,' vol. **25**, p. 55.
- Tarussov, B. (1927). 'Zurn. eksp. biol. med.,' vol. **6**, p. 229.
- Weil, E., and Pantin, C. F. A. (1931). 'J. Exp. Biol.,' vol. **8**, p. 73.

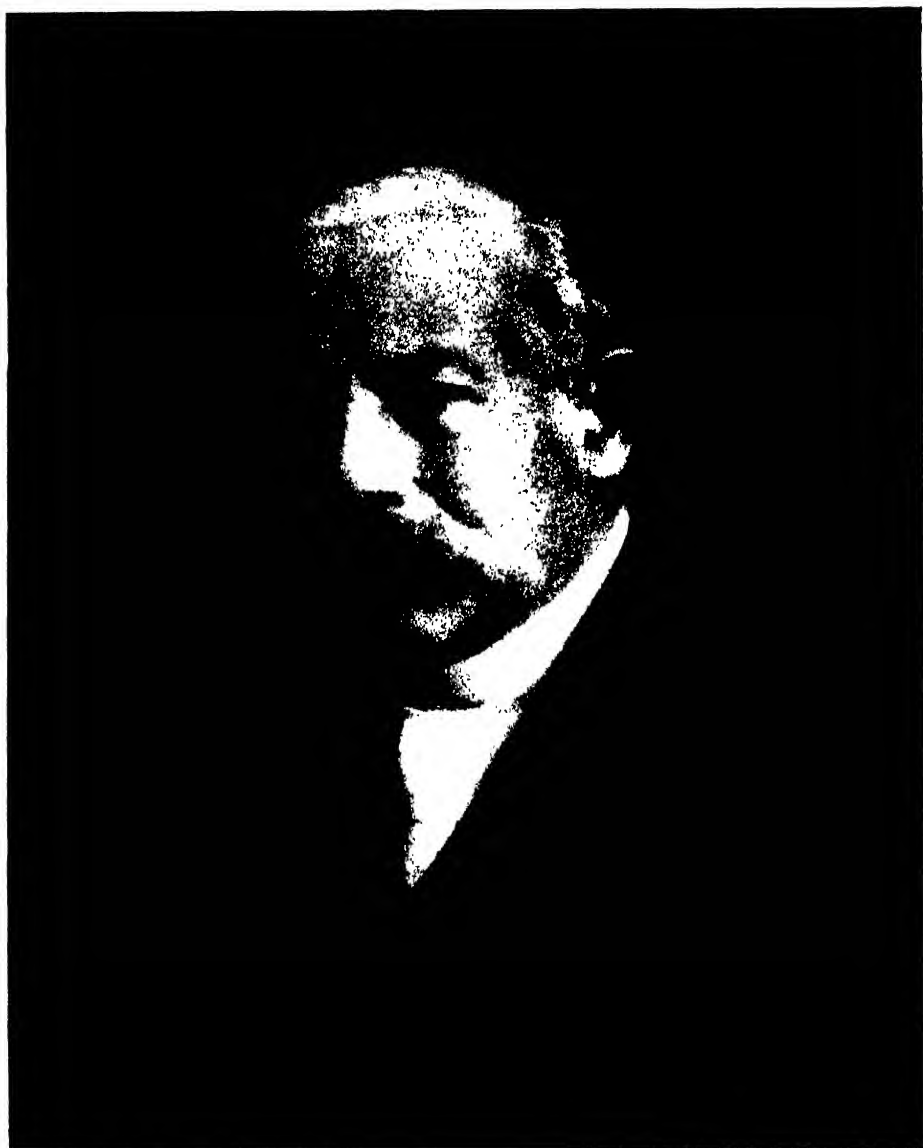
# OBITUARY NOTICES.



## CONTENTS.

	PAGE
HANS FRIEDRICH GADOW (with portrait) .....	i
THE EARL OF BALFOUR (with portrait) .....	iv
SIR WILLIAM BOYD DAWKINS (with portrait) .....	xxiii
JOHN WILLIAM EVANS (with portrait) .....	xxvii





*W. H. Shaw*

## HANS FRIEDRICH GADOW—1855–1928.

HANS GADOW was born in Pomerania on March 8, 1855, his father being the Inspector of the Prussian Royal Forests. He was educated at Frankfurt, Berlin, Jena and Heidelberg, coming under the influence of Ernst Haeckel and Carl Gegenbaur; the conceptions and methods of these two zoologists are evident in his earlier work, and indeed persisted throughout his life.

In 1880, he became an assistant in the Zoological Department of the British Museum, one of several men who, coming from foreign countries into the service of the museum about that time, subsequently attained distinction and influenced zoology in Britain.

In 1882, he was appointed Strickland Curator of Birds in the University Museum of Zoology at Cambridge, and two years later received the additional appointment of Lecturer in Vertebrate Morphology. In this year he became a naturalised British subject.

Only in 1920 was his lecturership raised to the status of a Readership. Gadow was elected into the Royal Society in 1892. He died in Cambridge on May 16, 1928.

Gadow was pre-eminently a Comparative Anatomist of Gegenbaur's school, one of the few men in England who was familiarly acquainted from his own dissections with the musculature, the gut and its derivatives, and other parts of the soft anatomy of a very large series of vertebrates.

This aspect of his work found expression in his classical monograph on the anatomy of Birds, in Bronn's "Klassen und Ordnung des Thier-reichs," an immense work of 1,000 pages and many plates. This book is marvellous in its completeness, even such minor characters as the "metallic" colours of feathers being discussed in it.

A small later volume on the classification of birds is founded on Gadow's encyclopædic knowledge of anatomy, and will always be valuable.

To such studies of a type which was familiar to Cuvier, Gadow added others in which he applied embryological and palæontological data to the solution of individual morphological problems. The most important of these were on the development of the vertebral column in Vertebrates, the evidence and conclusions appearing in two papers in the *Philosophical Transactions*. In them Gadow showed that, at an early stage, the vertebral column, whether of a fish or a reptile, consisted of a notochord and its sheath, together with four pairs of cartilaginous blocks in each segment. These, the arch cartilages or arcualia surrounded either the spinal cord or the dorsal aorta.

Subsequent work by many zoologists has confirmed the universal occurrence

of this condition, whose recognition by Gadow marked a turning point in our knowledge of the subject. Gadow suggested that this condition in the early development of every vertebral column provided an explanation of the peculiarities of the first two vertebræ of reptiles, and of the somewhat similar structures which extend throughout the column in the rachitinous labyrinthodont amphibia.

Gadow's account of the constitution of the adult vertebral column in living amphibia and in certain fossil forms was seized on by palæontologists, and now forms the basis on which the division of the class into orders rests.

Thus this work of Dr. Gadow, although it has proved incorrect in detail, has been most fertile, it has led not only to much embryological investigation, but has clarified the ideas of all students of vertebrate taxonomy.

In another paper in the *Philosophical Transactions*, on the modifications of the first and second visceral arches in Vertebrates, Dr. Gadow put forward a new solution of the problem of the mammalian auditory ossicles. This involved the transformation of the quadrate into the tympanic bone, and the transformation of the reptilian extra columella into the incus and malleus. Although the central thesis of this paper has now been disproved, it played its part in stimulating new investigation of the regions involved.

But Gadow's zoological interests extended far beyond morphology; he described an interesting case of meristic variation in turtle and used it in a general discussion of evolutionary theory, and he was greatly interested in the problems of animal colour and of geographical distribution.

In order to have first-hand knowledge of these matters, he travelled extensively in Spain and Mexico, observing amphibians, reptiles, and birds in their natural environments. The results are recorded in two books of travel, and in many papers.

In some ways, the best and certainly the most characteristic work which Gadow published was the volume on Amphibia and Reptiles in the 'Cambridge Natural History.' In this book, morphology holds a subordinate place, the greater part of it consisting of short and often most entertaining accounts of individual species regarded as animals living in the world.

It is full of observations of habits of all kinds—food preferences and the capture of food, locomotion, breeding habits, colour changes, the musical appreciation of Tortoises—many of them original, and most confirmed by his own observations of animals which he kept in his house outside Cambridge. Indeed, the whole book well displays the real love and understanding he had of these beasts.

Gadow's personal qualities endeared him to all with whom he came into contact. He invited and delighted in discussion, and was always willing to modify, even his favourite, morphological conclusions, if fresh evidence was brought to his notice.

The present writer first met him under characteristic circumstances. In 1912, the late Mr. R. W. Palmer had made a model of the lower jaw and auditory ossicles of an embryo *Perameles*, which so closely resembled the jaw of the fossil reptile *Diademodon*, as to make it obvious that the mammalian tympanic bone was the reptilian angular.

This heterodox opinion brought Gadow to his feet in support of his own beloved theory. He stated it in the form of a series of theses which he nailed to the door of the Zoological department in Cambridge, with a challenge to all the world to debate them on a certain day.

A public debate on these theses was duly held, in which I filled the part of villain. Ultimately, the new evidence became overwhelming, and Dr. Gadow withdrew his own hypothesis, soon forgiving my opposition, and adding me to that select list of zoologists who appear under nicknames in the marginalia which are thickly scattered over every pamphlet which he read.

Dr. Gadow filled a special place in British Zoology; he brought to us a conception of vertebrate morphology different to that which Balfour had so brilliantly developed in Cambridge. The influence of this attitude of mind has spread, not only and not so much through the students who attended his lectures, but to those others with whom he discussed morphological problems. It now, I believe, permeates much of the anatomical work which is in progress in Britain, and is still active in palæontology.

Thus Gadow's importance as a zoologist is probably greater than his published work, excellent though it is, would suggest.

But what remains in the memory of his friends is the charm of a most unusual personality.

D. M. S. W.

## THE EARL OF BALFOUR—1848-1930.

ANY attempt to summarise Lord Balfour's public career would be out of place in these 'Proceedings.' It has been thought suitable, however, to include a short memoir dwelling on his early history and mental development, on his scientific and philosophical thought, and on his administrative work for scientific, industrial and medical research.

Arthur James Balfour was the son of James Maitland Balfour of Whittingehame, Haddingtonshire (died 1856), and of Lady Blanche Gascoyne Cecil, second daughter of the second Marquess of Salisbury. The father was a country gentleman of fortune and of some ability, but in no sense a philosopher or a thinker, or even a reader. He served in Parliament, organised a regiment of yeomanry and was chairman of the North British Railway. His career however, was cut off at an early stage by consumption. The mother, Lady Blanche Balfour, came of an able stock. Although the influence of the great Lord Burghley must be considered to have been long since exhausted, the abilities of that branch of the Cecil family had been recently recuperated by two marriages: the first with Lady Mary Amelia Hill, first Marchioness of Salisbury; and the second with Frances Mary, daughter of Bamber Gascoyne, and mother of the third Marquess, Prime Minister to Queen Victoria, and of Lady Blanche Balfour. Lady Blanche shared to a marked extent her brother's ability. She directed the education of her children, and found means of stimulating their intellectual interests, with the greatest tact and judgment. She was particularly judicious in the choice of the books which she read to them. On occasion she would cut out passages which were tedious or unsuitable, and substitute connecting links rewritten by herself.

Her lessons in arithmetic are remembered for the clear way in which the subject was presented. The reasons for the operation of "carrying," *e.g.* were lucidly explained, in contrast to the usual dogmatic method.

Although Lady Blanche's own tastes were literary rather than scientific, she successfully directed the attention of her children to various branches of natural history, and, in the case of some of them, the interests thus aroused bore no inconsiderable fruit. Thus Gerald and Francis Balfour wrote an account of the local geology of East Lothian, which has permanent value as a contribution to the subject. Francis Balfour, it is hardly necessary to remind the reader, eventually became Professor of Animal Morphology at Cambridge, a chair specially created for him. Those best qualified to judge held that his early death represented an irreparable loss to science.\* His

\* It has sometimes been imagined that Arthur Balfour derived his knowledge of science and his interest in it at second hand from his brother Frank. I am sure that no one with inside knowledge would share this view. The taste was innate in both of them, and came out in both, though in widely different forms.



✓ NoJa up





youngest sister, Alice Balfour, assisted him to some extent in his studies, and has ardently continued the pursuit of local entomology up to the present. Arthur Balfour's eldest sister had a distinct gift for mathematics and physics, and in later years was able to render valued assistance to her brother-in-law, Lord Rayleigh, during his tenure of the Cavendish Professorship of Experimental Physics at Cambridge. She appears as joint author of some of his papers on Absolute Electrical Measurements.

It will be seen then that there was a marked scientific tone in the family of which Balfour was a member. The same was traceable in his uncle, Lord Salisbury, who to some extent occupied his leisure with botanising, and with experiments in electricity and magnetism, which he carried on in a private laboratory at Hatfield. Balfour seems to have taken less part than some of the others in these natural history studies. He had, for instance, no inclination for the hard work of a systematic search for fossils. But, no doubt, what was going on had its influence upon him.

In later life he took some practical interest in forestry on his estate, but he had no knowledge of horticulture, and no eye for bird life. On one occasion, well remembered in the family, he remarked that he had been disturbed by a bird which had got into his study. His youngest sister, alive with the instincts of a naturalist, wished to know what species it belonged to. But the only description he could give was "Oh, I don't know; average small bird." Although he had not this kind of knowledge himself, he respected it in others. As a young man, he had shooting, deer stalking and salmon fishing at his disposal, but he soon abandoned them in favour of lawn tennis and golf.\*

Balfour first went to school at the Rev. C. G. Chittenden's at Hoddesdon. His opinion of the school was expressed to the parents of another small boy in the words, "Send him to Chittenden's. It is the only place where I ever learnt anything." Mr. Chittenden, when asked who was the ablest pupil he had had, replied "Arthur Balfour," and, although this judgment was given after his quondam pupil had made a public reputation, no doubt he had been of the same opinion throughout. Mr. Chittenden was a man of wide general information, and an interesting talker, though a stern disciplinarian in school hours. Master and pupil seem to have had a warm regard for one another, and after the latter had left for Eton, it was Mr. Chittenden's favourite relaxation to visit him there. While at the school Mr. Chittenden often took him out for walks. An interest in music was one that they had in common, and it is probable too that they discussed scientific topics, with which Mr. Chittenden had some acquaintance. It is certain at least that Balfour dipped

\* A fishing exploit at a very early age is remembered, however. He had succeeded in landing an eel, and was executing a dance in celebration of his victory with such vigour that he danced into a bed of stinging nettles. His yells of triumph soon gave place to yells attributable to a very different cause.

into various branches of science at this time, though perhaps no more deeply than many boys do. The present writer possesses a copy of "Carpenter on the Microscope," with the inscription "A. J. Balfour, April, 1860, Eton College." Balfour is remembered to have brought home a frictional electrical machine, and to have made some attempts, though apparently without special tenacity or success, to carry out experiments in electrostatics, with home-made accessories.

As we have seen, he had impressed Mr. Chittenden, and at Eton he similarly impressed William Johnson,\* who seems to have shown more discernment than most of Balfour's masters or contemporaries at this stage. His intellectual development was not precocious, but, in the event, it went on much longer than that of some of his early friends, who had, for a time, seemed to stand on a level with him. Indeed, it may be said that he went on developing almost to the end.

It has sometimes been thought that Balfour, like his uncle, Salisbury, found the atmosphere of Eton uncongenial. I am sure that he never said anything of the kind in my hearing: on the contrary he pressed strongly for some of his nephews to be sent there with the words "Much the best school." Nor was he disposed at all definitely to condemn the classical system of school education, though he was himself included in that large majority who, after spending years under the system, fail to acquire a working knowledge of the classical languages.

During the Lancashire cotton famine of 1862-63, Lady Blanche Balfour conceived the idea of saving money for the help of the distressed artisans, and incidentally providing a valuable practical experience for her young family by domestic economies. A projected trip to the continent was abandoned, and the household at Whittingehame was much reduced, the family helping with the housework. During the summer holidays Arthur Balfour and his brothers made the beds and blacked the boots, while his sisters did the cooking.

The time approached for him to go to the university. He had not made much progress in, or shown aptitude for mathematics, and it was necessary to make up for lost time. His sister, Eleanor (Mrs. Henry Sidgwick), remembers reading elementary trigonometry with him for the "Little-go." They had to make it out from a book as best they could without the help of a tutor, getting up early in the morning, and fortifying themselves with bread and milk for the effort. When they came to the point that  $\sin \theta/\theta$  has the limiting value unity as  $\theta$  is indefinitely diminished, Arthur Balfour was dissatisfied with the demonstration in the book (probably not without reason according to modern standards of mathematical rigour). But he thought the point would be interesting if one could fully understand it.

In later life he was deeply interested in the philosophical foundations of

\* Later known as Cory.

mathematics, particularly in connection with the theory of probability, and deplored that he had not the technical knowledge to follow current developments either in that direction or in mathematical physics. He often regretfully commented "I expect it is too mathematical for me."

Within the family circle, he took his place as the leader in intellectual interests. He it was who usually discovered to the others new avenues in literature. For instance, he came back from school on one occasion brimming over with interest in "Goethe."

The social tact which distinguished him in later life was innate, and already apparent in his boyhood. He was somewhat careless in the matter of dress, and very much detached from the smaller anxieties of everyday life. For example, he was driving with his eldest sister to a dinner. She expressed doubts as to whether the coachman was going the right way. "That," said Balfour, "is his affair."

He went up to Trinity College, Cambridge, in the October term of 1866, as a fellow-commoner according to the custom of those days, as his father had done before him. This gave him the doubtful privilege of wearing a gown embroidered with silver,\* and the valued one of sitting at the High Table with the dons. Here he was brought into contact with Henry Sidgwick and John Strutt (afterwards Lord Rayleigh) who were a few years senior to himself, and fellows of the college. With them he formed an enduring friendship, which in each case developed into something more. For the former eventually married his eldest sister, Eleanor (1876), and the latter his second sister, Evelyn (1871).

It does not appear that Arthur Balfour impressed his individuality very strongly on the High Table at Trinity. Rayleigh remembered a discussion there, a few years later, as to which of the Balfour brothers had the most ability. Some were for Gerald, others for Frank. When he himself put in a claim for Arthur, the general opinion seemed to be that he was propounding a paradox. There were no doubt substantial reasons for awarding the palm to his younger brothers at that time. They had achieved high academic success, whereas Arthur Balfour did not rise above the level of a second class in moral science. His tutor, Henry Sidgwick, who, with Rayleigh and Rayleigh's younger brother, Charles Strutt, had formed the highest opinion of him, was disappointed, but not altogether surprised by this result. Balfour was also somewhat disappointed himself, though academic success had not been a prominent aim in his mind. The explanation seems to have been that he had paid too much attention to the current problems of philosophy, and not enough to its literature and history.

The truth is that his was a mind which could ill submit to the bondage of following a prescribed course of study. With him the motive must be his

\* He is said to have been the last, or almost the last fellow commoner.

own personal interest, not the fulfilment of a task. That was and remained repugnant to him whether an academic text book or the text of a parliamentary Bill was to be assimilated. In later life he several times gave public expression to his dislike of the tyranny of the examination system, though he did not pretend that it could be avoided.

Frank Balfour, who came up to Cambridge in 1870, took on the college rooms (A 4, New Court) which had been occupied by his elder brother, and was thus served by the same bedmaker. Adam Sidgwick, the zoologist, who was a devoted disciple of Frank Balfour, was fond of quoting her observation that "Mr. Arthur Balfour left a great many books about, but Mr. Frank read them through." Arthur Balfour had, in fact, an extraordinary faculty for getting hold of the essentials of a subject without apparently feeling the need for systematic study. Later in life when casually asked how long he could continue reading a stiff book, he put the limit, rather paradoxically perhaps, at ten minutes.

The following remarks\* express his own point of view.

"We misuse the word superficiality, I think; sadly misuse it. Superficiality does not depend upon the amount of knowledge acquired. It is a quality rather of the learner than of the thing learned. The smallest amount of knowledge may be thorough in the sense in which the word should be used. Knowledge of the general principle may be obtained by those who have neither the time nor the ability to master the details of any particular branch of science; but to say that that smaller modicum of knowledge is therefore superficial, and therefore useless, is wholly to mistake what superficial knowledge consists in, and what education aims at. You may know very little, and not be superficial; you may know a great deal, and be thoroughly superficial. Superficiality is a quality of yourselves, not of the knowledge you acquire."

It was at about this stage of his career that he read Darwin's "Origin of Species," and its effect on his point of view was profound. His own mentality, it is true, was in many ways very different from that of Darwin. He had no store of detailed systematic knowledge, and it would probably not have been congenial to him to acquire it, or himself to attempt to sift the wheat from the chaff. But any one who reads the earlier chapters of the "Foundations of Belief" will see how much he had been influenced by the study of Darwin. Such reserve as he had was not founded on detailed criticism of Darwin's facts or methods of reasoning. The following quotation† will illustrate its nature.

"It is wrong to suppose that these supreme values [*i.e.*, what is highest and

\* Speech at the opening of New Hall of Battersea Polytechnic, February 3, 1899 (Times). Reprinted in "Arthur James Balfour as Philosopher and Thinker," selection by Wm. Short 1912.

† 'Theism and Thought,' p. 28.

rarest] in æsthetic ethics and thought seriously count in the struggle for existence. Saints, philosophers and artists have never, so far as I know, been specially successful in rearing large families themselves ; nor have they enabled the communities which admired and occasionally produced them to crowd out rival populations from the rich places of the earth. As Nature measures utility, they are useless. In no effective fashion do they make for survival. They are but casual excrescences on the evolutionary process forming no part of its essential texture. They are, on the naturalistic hypothesis, an accident of an accident."

" Few things on the spiritual side of evolution are more interesting than this. It is not perhaps strange that the onward momentum of those developments which make for biological success should carry them into regions where all, or almost all, their survival efficiency vanishes away. But surely it is strange that they or something of them should acquire new and higher values which naturalism can hardly explain and certainly cannot justify."

Balfour throughout life had the highest admiration for Darwin, " because," he said " he was not a partizan—he really wanted to find out the truth—an attitude of mind seldom found among men of science, and never among theologians."

This opinion was not formed without the knowledge that comes of personal contact. He had been introduced to Darwin's home at Downe by the latter's son, George, who was one of his early friends at Cambridge, and to whom he remained warmly attached to the end.

Shortly after taking his degree, Balfour went with his friend, John Strutt, to visit the Gladstones at Hawarden Castle. Rayleigh often referred to an incident on this visit which evidently produced a strong impression upon him. For some reason it was necessary to ease the labours of the household, and in consequence Mr. Gladstone took Balfour and Strutt to dine at the village inn. Probably the two former took the chief part in conversation, and Strutt, it is likely, was a comparatively silent onlooker ; be that as it may, he presently perceived that Balfour was not taking the veteran statesman seriously, but was amusing himself with a psychological study, and, as he often related the incident afterwards, " was playing Gladstone like a fish." Balfour's age at this time, be it remembered, was only twenty-two years.

The circumstances which led to Balfour's entering on a political career are explained in the fragment of autobiography, which will appear shortly. We are here rather concerned with the question of why he did not take up science. The following extract from his sister Evelyn, Lady Rayleigh's journal of September 10, 1888, will explain his own point of view :—

" Yesterday A. was as usual expressing a wish that he had been a scientific man instead of a politician."

E. "Why did you not devote yourself to science then?"

A. "I did not consider I was capable of it."

E. "John\* does not agree with that, he says a conversation on science with you sometimes strains his powers."

A. "Oh, nonsense—but I was too lazy, I should never have had patience for the drudgery."

It is perhaps scarcely worth while to devote much consideration to what *might* have been. Other observers will probably agree with Balfour's own view that attention to drudgery was not his strong point. At the same time it is difficult to believe that he can have got through so many years of successful public life without having done a good deal of it.

He was certainly not an enthusiastic politician. He remarked on one occasion (1893) that his mind did not naturally run to politics. He never thought of them in bed, which was the test. He regarded them with a calm interest, but as for getting excited over them as some people did—he could not do it.

He was, I think, comparatively indifferent to both the trials and the rewards of official life as a party politician. He did not often trouble to inform himself as to what people were saying about him, and he seldom looked at a newspaper. He remarked that everything conceivable had been said of him, good, bad and indifferent, and that in view of this was difficult either to be much depressed or much elated by the sum total of the opinions expressed. He may have been somewhat hurt at having temporarily lost the confidence of the Unionist Party in 1911, but if this may be suspected it is not from anything that could be seen at the time, but from slight indications elicited by later events.

His detached attitude towards criticism may perhaps have made the sheltered life of a student relatively less attractive to him than to many others equally well qualified to pursue it. As to the honours and rewards of a public career, I know on the best authority possible that he accepted most of them with reluctance, and because circumstances made it difficult to do otherwise.

Finally, it may be remarked that most people realise more clearly the disadvantages of the career they have actually adopted than of the one they might have adopted and did not.

Balfour was first elected to Parliament as member for Hertford in 1874, and from this time on became increasingly immersed in public activities which do not fall within the scope of this notice; at no time, however, were his intellectual interests allowed to fall altogether into the background, and his first speculative work, "A Defence of Philosophic Doubt," appeared in 1879, shortly after his return from the Berlin Conference of 1878.

\* Lord Rayleigh.

The title was not chosen without careful consideration, but none the less its meaning was widely misunderstood. As anyone who looks even casually into the book may see, doubt about the views of Mill and Spencer is advocated, not doubt about popular theology. "It appears," he says, "that the practical conclusions I draw from a sceptical philosophy have little or no tendency to alter the internal structure of any actual or possible creed."

The "Defence of Philosophic Doubt" discusses in turn each of the various theories of knowledge which at the time of writing could be considered to have any important following. J. S. Mill, Spencer, Kant, Hamilton, and the agnostic scientific school represented by writers like Huxley and Leslie Stephen, are each subjected in turn to a critical analysis, which is carefully limited to essentials. The author is never led into controversial bye-paths, however tempting, in order to score a point. He concludes that not one of these schools of thought is self-consistent, though he does not claim to be able to produce anything better. At the same time he carefully explains that he, like everyone else, cannot help accepting in practice the methods and conclusions of science, in spite of the incoherence he finds in them, regarded as a logical system. As a "practical result" he recommends that scientific conclusions should be provisionally adopted alongside of theological ones, even at the cost of apparent inconsistency. I do not know of any evidence that other thinkers at that time found themselves able specifically to accept this recommendation. It must be admitted, however, that an attitude very like this has been taken up in modern science, in using the wave theory of light to co-ordinate one set of phenomena, and the corpuscular theory to co-ordinate another set.

The general line of argument in this book has much in common with Balfour's second and better-known book, the "Foundations of Belief," on which he placed, perhaps, a higher value.

About the time of publication (December, 1894) he said, in intimate conversation, that he felt he had a message to give, which he was trying to give in this book, and which was of far greater importance than anything he had done or could do in politics. He was especially pleased and encouraged to find that his brother-in-law, Henry Sidgwick, though not always in agreement, thought highly of it; and as the public were anxious to have the views of a conspicuous public man on questions of such fundamental interest, the book sold largely.

In this, as in the author's other philosophical works, the greater part of the text is occupied with argument destructive of the philosophical point of view which he refers to as Naturalism, and, so far as I have been able to gather, it is this sceptical criticism, carried out with a wealth of illustration from the scientific field, and an easy dialectical mastery, which leaves the most marked impression on the minds of the generality of readers. As a brief example of the



method we may quote\* "Though we are quite familiar with the fact that illusions are possible, and that mistakes will occur in the simplest observation, yet we can hardly avoid being struck by the incongruity of a scheme of belief whose premises are wholly derived from witnesses [the physiological mechanism of the senses] admittedly untrustworthy, yet which is unable to supply any criterion, other than the evidence of those witnesses themselves, by which the character of their evidence can in any given case be determined."

The author, however, was most anxious that the constructive part of his argument should not be overlooked, or misunderstood.

"I seem" he wrote† "to have given certain of my critics the impression that the principal, if not the sole object of this work, was to show that our beliefs concerning the material world and those concerning the spiritual world are equally poverty stricken in the matter of philosophic proof, equally embarrassed by philosophic difficulties. This, however, is not so . . . The dissipation of a prejudice, however fundamental, can at best be but an indirect contribution to the work of philosophic construction. Concede the full claims of the argument just referred to, yet it amounts to no more than this—that while it is irrational to adopt the procedure of naturalism, and elevate scientific methods and conclusions into the test of universal truth, it is *not* necessarily irrational for those who accept the general methods and conclusions of science to accept also ethical and theological beliefs which cannot be reached by these methods, and which, it may be, harmonise but imperfectly with these conclusions. This is indeed no unimportant result; yet, if the argument stopped here, it might not be untrue, though it would assuredly be misleading, to say that the following essay only contributed to belief in one department of thought by suggesting doubt in another. But the argument does not stop here. The most important part has still to be noted—that in which an endeavour is made to show that science, ethics, and (in its degree) æsthetics, are severally and collectively more intelligible, better fitted to form parts of a rational and coherent whole, when they are framed in a theological setting than when they are framed in one which is purely naturalistic."

During the interval between his retirement from the leadership of the Unionist Party in November, 1911, till the outbreak of war, Balfour had, perhaps, more leisure for intellectual pursuits than at any other period of his mature life, and a near observer described him as like a bird which had escaped from a cage. To this period belongs the photograph reproduced herewith,‡ the holiday attire in which he appears harmonising with his mood at the time. This interval of comparative leisure was partly employed in preparing the first series of Gifford lectures delivered at the University of Glasgow in

\* "Foundations of Belief," 8th ed., p. 118.

† "Foundations of Belief." Introduction to 8th ed., pp. xvii, xviii, 190 l.

‡ Taken by Admiral Strutt in the conservatory at Terling.

January and February, 1914, and published under the title "Theism and Humanism." The general scope and mode of treatment is not unlike that adopted in "The Foundations of Belief." Their success was extraordinary; the audiences amounted to something like two thousand and increased beyond the limit of seating accommodation as the course of ten lectures proceeded. He did his best to avoid technical language, which he considered was very apt to mask a confusion of thought; it was impossible however to avoid words like *empirical* and *a priori*. Two ladies were heard discussing who this *a priori* might be—some Italian philosopher they supposed. However, in spite of rather discouraging symptoms of this kind, he hoped and believed that most of his audience carried away something.

The services which Balfour's party had so easily dispensed with in times of peace were found necessary by his country in the hour of stress, and he was called to be First Lord of the Admiralty in May, 1915, shortly before the lectures were actually published. His brief interval of comparative leisure was at an end.

The second course of Gifford lectures was necessarily deferred till after the war. It was delivered in 1922-23 and published under the title "Theism and Thought."

Balfour, like many of his relatives, felt a sympathetic interest in psychical research. Some of his political followers were disposed to complain of this. They classed it with Bi-metallism and Female Suffrage, and considered that all these "fads" injured his position as a leader. When criticism of this kind came round to him, he said that he was not prepared to give up his "fads," and that if a choice was necessary he would sooner abandon politics.

In 1894 he gave a presidential address to the Society for Psychical Research.\* In this, among other topics, he emphasised the warning to be taken from the incredulous attitude of the scientific world towards hypnotism. "There were, indeed, a good many doctors and other men of science who could not refuse the evidence of their senses, and who loudly testified to the truth, the interest, and the importance of the phenomena which they witnessed. But if you take the opinion of men of science generally, you will be driven to the conclusion that they either denied facts which were obviously true, or that they thrust them aside without condescending to submit them to serious investigation."

Balfour was, I believe, convinced of the reality of telepathy, and his conviction was fortified by some casual experiments with Prof. Gilbert Murray in which he took a personal part, and which excited a good deal of attention in the newspapers. On the alleged physical phenomena of spiritualism he had an open mind. He was, for example, unable lightly to dismiss the concurrent testimony of the late Lord Crawford and the late Lord Dunraven to some of the most marvellous happenings. He had known these men, and

\* Reprinted in "Essays, Speculative and Political," 1920.

respected their capacity and good sense. Pressed to sum up, he answered, in a truly scientific spirit, that more experiments were needed.

As I have endeavoured to show, Balfour's knowledge of the essentials, rather than of the details of contemporary science, was wide. When he was called upon to speak publicly on scientific or semi-scientific questions he was usually able to illustrate a topic or to choose one from his own knowledge. Thus, when he was President of the British Association at Cambridge in 1904, he delivered an address in which what were then novel views of the electronic constitution of matter were discussed in their philosophical aspect. This was perhaps the first occasion when emphasis was laid before a popular audience on the glaring discrepancy between the new ideas of the atom, with its relatively vast inter-electronic spaces, and the old philosophic distinction which made shape a "primary" property of matter, existing independent of the observer, while secondary qualities, such as colour, were thought to have no such independence. It is not, I think, an exaggeration to say that these conceptions are found novel and illuminating by many educated people even now, more than a quarter of a century after the address was delivered.

Foreign savants who attended as guests of the Association were a good deal astonished at the range of scientific knowledge of the speaker, and some of them were disposed for a moment to doubt whether he could really be the Prime Minister, in part because he wore no decorations. After the opening meeting Balfour attended some of the sectional proceedings, in which he was able to take part. No doubt at times he used the arts of the experienced public man in making a necessarily limited knowledge go as far as possible. But he was always eager to learn more.

An incident remains in my mind as an illustration of this: it was, I think, during the autumn of 1928. We had been out for a country walk together, perhaps almost for the last time, and after the conversation had ranged over a great variety of topics including the morals of the present generation (which he did not think really worse than those which prevailed during his youth), the distribution of honours, the prospects of future taxation, and the literature of the eighteenth century, it came round, I do not remember exactly how, to the electromagnetic theory of light. "There are things I sometimes talk about," he said, "which I find it very difficult to get any grasp of. I think I understand pretty well the relations between electric currents and magnetism, but I cannot really form any conception of Maxwell's theory of the propagation of electromagnetic waves." I said I thought it was too difficult for general treatment. Maxwell's calculations must be followed through in order to get any insight into it.

He declined, however, to be put off in this way, and insisted on my trying to expound it. I did what I could, helping myself out with diagrams drawn on the road with a walking stick. However poor the attempt, he seemed fascinated

with the subject, and I remember trying to think of anyone else who would have been able to learn anything from such an explanation.

Relativity, occupying as it does the borderland between science and philosophy, interested him deeply. One remark he made on this subject is of some psychological interest, whether one is able to agree with it or not. He complained that the popular expositors put an undue and unnecessary stumbling block in the way of their readers when they traced out the paradoxical consequences of extreme suppositions, such as observers travelling on projectiles which moved with the velocity of light. I tried to plead that in science hard cases made *good* law, but he was not to be moved from his position. As regards the technique of physical measurements he was not so much interested. He could, of course, appreciate well enough the necessity of reaching a certain standard of precision in order to resolve a particular problem in hand; for instance, the standardisation of machine parts to go together without fitting. But he was not prepared to regard improved accuracy of measurement as an end in itself. This was evident enough to those who had the opportunity of discussing scientific subjects with him. Others will find an illustration of it in a reference to the measurement of solar parallax by the transit of Venus in one of his essays.\*

Balfour was elected into the Royal Society under Statute 12 as early as 1888, and served on the Council in 1907-08 and again in 1912-14. In 1920 when the question of an election to the Presidency came up, the retiring President, Sir J. J. Thomson, was commissioned by the Council to find out whether Balfour would allow his name to be put forward, on the understanding that the Society would want him as an active, and not merely an ornamental president. Balfour was more than commonly pleased to receive so great a mark of the confidence of the scientific world; but he was already President of the British Academy; as Lord President he was responsible for the Department of Scientific and Industrial Research; he was taking a very leading part in the affairs of the League of Nations; and in addition to all this the Cabinet was meeting once, twice, or even three times a day. He felt that to add to this programme of work was impossible.

We have seen how Balfour had acquired a far wider scientific culture than usually falls to the lot of practical politicians. This culture had helped his philosophical studies, and had enabled him with the greater force to give public expression to his belief in the national importance of scientific pursuits. But otherwise it had not had much constructive outcome; nor can it have seemed likely in 1919 that at the age of 71 years it would ever do so. His political career seemed for the moment to be closing, and he was determined not to do any more heavy political work of the ordinary kind—leading the House of Lords, for example. He had always been inclined to rate rather low the value

\* "*Essays and Addresses*," 3rd ed., 1905, pp. 23-24.

of what could be accomplished in that way\* and he felt that he had earned his rest from it. Yet his public career was about to enter on a new phase, in which his scientific interests and acquirements were to bear no inconsiderable fruit. This phase seemed to himself more interesting and at least as important as any that had preceded it.

It is necessary to go back somewhat in time and to recall that in connection with Mr. Lloyd George's National Health Insurance Act of 1911, a large scheme of medical benefit was supplemented† by a considerable provision for medical research. Balfour spoke strongly in favour of this in the House of Commons, but the decision had apparently been taken already, and I have not been able to learn that he was definitely instrumental in bringing it about. The Medical Research Committee was the outcome in 1913, and this in 1919 became the Medical Research Council, under a Privy Council Committee and the Lord President. The war led to a great stimulus of State-supported science; for scientific problems arose in connection with almost every war activity. One may recall aeronautics, radio communications, anti-submarine work, poison gases, medical and surgical war problems such as antitetanus inoculation and plastic surgery, sound ranging—but the list is almost inexhaustible. In this way politicians and administrators were brought into contact with science and with scientific men. The ice was broken; budding scientific institutions underwent a forced growth; and when the war was over its lessons could not be unlearned, and the growth which had taken place proved on the whole to be permanent. It is a significant fact that when the post-war *regime* of retrenchment set in, it was not extended to research activities.

The Department of Scientific and Industrial Research was founded in 1915 and placed under the Lord President of the Council. Many new State scientific activities have been placed under the department—fuel research, low temperature research, building research, forest products research, chemical research, radio research, and two long-established scientific institutions, the Geological Survey and the National Physical Laboratory were also annexed to it.‡ The department has many other responsibilities besides these. It

\* Extract from Lady Rayleigh's journal, June 16, 1892 :—" Paderewski was at the Royal Society Soirée last night, and in discussing it A. remarked of the scientific guests, ' They are the people who are changing the world and they don't know it. Politicians are but the fly on the wheel—the men of science are the motive power.' " The same point of view is more elaborately set out in his essay, " A Fragment on Progress," republished in " *Essays and Addresses*," 1905. See particularly pp. 260-262.

† Probably on the initiative of the late Sir Robert Morant, K.C.B., of the Education Office.

‡ The latter had been founded in 1900. Balfour was then First Lord of the Treasury, and was most sympathetic to the scheme. Indeed the suggestion that the laboratory should go to Bushy Park originated with him. War requirements had increased the extent of the laboratory's operations out of all knowledge, and it had become impracticable for the Royal Society to continue to carry the liability for balancing income and expenditure.

administers large funds for research purposes in academic institutions and industrial research associations.

Balfour, after his return from Paris in 1919, retired from the Foreign Office, in accordance with his determination to do no more heavy political routine, and undertook the lighter duties of Lord President. He then found himself in possession of the heritage which has just been sketched in outline. He probably had little up-to-date information about his new responsibilities, for since 1915 his time had been fully occupied at the Admiralty and the Foreign Office, and he cannot have been able to give much attention to anything else. One evening in the autumn of 1919 the Secretary of the Medical Research Council, who was somewhat pressed with work, was told that there was a gentleman to see him—Mr. Balfour. "I did not make any appointment, did I?" he said. "I do not think I can see him. What Mr. Balfour is it?" To his astonishment it turned out to be the Lord President. It was quite a new thing in his experience that a Minister should call on an official who served under him, and he had no reason to think that previous Lord Presidents had even known where the office was. Balfour opened the conversation by remarking that his new appointment seemed to give him very little to do, but that he was delighted to find that he was the head of two research organisations. He had come to learn whether there was anything he could do to help.

It soon appeared in practice that in those troublous times other ministerial duties and distractions made far greater claims than he had anticipated, and during the period of office from 1919 to 1922 when he went out at the fall of the Coalition Government, he was not able to attend the meetings of the Medical Research Council. As responsible minister he made no trouble about details. When formal matters were put before him he was content with the assurance of his advisers that all was right, and initialled them without further comment. "And now," he would say, "tell me something interesting."

For a short interval between 1922 and 1924 Balfour's connection with the Council was intermitted.

Lord Curzon became Lord President in 1924 and when Lord Irwin, who had been chairman of the Medical Research Council, went to India in 1925, Curzon sent to ask the advice of Balfour, who was not then in office, as to who should succeed him. It soon appeared that he was willing to serve himself, and so it was arranged. He became chairman, and attended every meeting. If he had a fault, it was not one that would be expected in a political chairman. He was if anything too prone to spend time at the council in discussing the scientific rather than the administrative aspects.

On Lord Curzon's death in 1925 Balfour again became Lord President, at Mr. Baldwin's special request. The problem then presented itself of whether

he could continue chairman of the advisory body, whose duty it was to give advice to himself.

The Council did not wish to lose him and arguments were readily found to justify the course which everybody desired should be taken. In the event he remained chairman until his last illness in 1930.

Balfour followed the developments of medical research with keen interest. He had known in his youth Sir James Simpson, of Edinburgh, the first to use chloroform as an anæsthetic, who was an intimate friend of Lady Blanche Balfour. He had early become acquainted with the methods of preventive inoculation by killed bacterial cultures by Sir Almroth Wright, to which he had become a firm convert. During the most active part of his Parliamentary career he was frequently placed *hors de combat* by feverish colds. It was often thought that these illnesses were merely diplomatic, but the suspicion was unfounded. He tried preventive inoculation, and benefited greatly from it. In this way he was brought into contact with the leading worker on the subject and his scientific interest was aroused. His friends and relatives heard a great deal at that time about streptococci and staphylococci. On the question of ordinary vaccination against smallpox his views were somewhat heterodox. In the autumn of 1898 he said in conversation with an intimate circle that he had been in favour of inserting a conscience clause into the Vaccination Act recently passed, and that since the Bill had passed he had looked somewhat further into the evidence, with the result that he had grave doubts whether vaccination was of any use at all. It was, he said, a question of statistics, not of science. In 1802, when vaccination was introduced, smallpox had decreased enormously, but much of the decrease had taken place, it was found, before vaccination became general. In 1876 when the belief in vaccination was at its height we had an epidemic of smallpox which threw into the shade the worst epidemics of the eighteenth century. There were, he said, other facts of a similar character.\*

During Balfour's chairmanship of the Medical Research Council he did not fail to attend the occasional afternoon teas at the Council's Institute for Medical Research at Hampstead, when workers such as Gye and Barnard gave an account of the problems on which they were engaged. He took every opportunity of pointing out in occasional speeches the debt which the nation owes to medical research workers and the danger of allotting too large a share of the nation's gratitude and support to those who apply medical knowledge, to the neglect of those who originate it in the seclusion of the laboratory, far away from the public eye.

The Department of Scientific and Industrial Research benefited not less than the Medical Research Council from Balfour's connection with it, which

\* These remarks are from a contemporary record. The present writer is not qualified to offer any comment upon them.

likewise began in the autumn of 1919. Here also he was in the habit of attending council meetings, sitting next to the chairman, Sir William McCormick, with whom he was on friendly terms. Indeed, friendliness was always the note in his relationship with civil servants, whether high or low. Thus, after official interviews at Balfour's house in Carlton Gardens, the secretary of the Department was commonly asked to stay to lunch, and the subject of discussion was often carried further in Balfour's family circle. The secretary's secretary was known to remark that during her official experience she had only twice been personally addressed by a Cabinet minister. The first time by one who shall be nameless, and who said "You may go"; the second time by Balfour, who said "Pray don't go, pray don't go."

To return however to the meetings of the advisory council. Balfour's contributions were mainly in the form of questions, which often opened a new point of view to the Council, and always referred to essentials.

His policy did not always err on the side of caution, and he was on occasion prepared to go beyond the advice of his Council. Thus the fuel research board which is under the department had been concerned with the hydrogenation of coal. There was the opportunity of purchasing for £35,000 ten years of experience on the problem, a half-scale plant, and all necessary opportunities for acquiring the knowledge to work it. The Council were not enthusiastic, some of them who were best qualified to express an opinion thought that the possibility of practical results was remote, and that the money from the limited budget would go further in other ways. However, a resolution of cold approval was eventually passed, and Balfour decided that the possibilities were so important from a national point of view that the expenditure was a legitimate gamble.

Again, when difficulty was anticipated in financing the promising work of the magnetic laboratory at Cambridge, under Dr. Kapitza, rather than that the work should be hindered, he was prepared to shoulder the responsibility of financing it from the department. This might be held to be somewhat irregular, but he expressed his willingness to defend it in Parliament. Had he been called upon to do so, his dialectical resources would, no doubt, have proved quite adequate to the occasion.

Balfour's attendance at the Council meetings, and his afternoon calls—unannounced and without appointment—at the secretary's office, were found most stimulating and encouraging. On the latter occasions, he would sit in an armchair discussing difficulties and hopes of achievement. He would ask what the truth really was about matters which were less ripe. Often the director of fuel research and other technical officers would be called in to the discussion, and the same sense of stimulus was experienced by them.

During Lord Curzon's term of office as Lord President, the question had



been mooted of devising machinery for co-ordinating the work of different government departments, and bringing the activities of the Research organisation now grouped under the Privy Council into more effective relation with them. The initiative came from members of the civil service. Lord Curzon's attitude was not very sympathetic at first, and further discussion was cut short by what proved to be his last illness. Balfour, as chairman of the Medical Research Council, was conversant, as Mr. Baldwin well knew, with these preliminary moves, and when he succeeded Curzon in office as Lord President (1925) Mr. Baldwin commissioned him specially to take up the problem.

One suggestion was to appoint a standing committee of leading scientific men. Balfour said "No. In the first place you are putting an undue burden on them. Secondly, you will have to make an invidious choice as to whom you ask to join the committee, and any man you select will be adapted perhaps to one problem, and not at all adapted to another. It would be far better to have a more elastic system and to imitate the organisation of the Committee of Imperial Defence."\* Distinguished civil servants who had been called into consultation were soon won over to see the wisdom of this point of view.

The Committee of Imperial Defence, the reader may be reminded, was of Balfour's own contrivance. He devised it in 1904 when he was Prime Minister, because he found that no attempt was being made by the departments to pool their information, or to arrive at clear mutual understanding about war problems that might affect more than one of them. Thus the Navy said that *they* made us safe from invasion, while the Army said that we were in the greatest danger because *they* were not maintained at a sufficient strength; and the Post Office had no idea that *they* had anything special to do in war, though in fact the part they have to play is very important. And similarly in other cases.

The organisation originally set up by Balfour in 1904 to remedy this state of things is a committee, with only one permanent member—the Prime Minister. The other members are such persons as he may summon to sit—mainly his colleagues in the Cabinet, but also the heads of the fighting services, and occasionally other experts. But this is only the first stage. The detailed

\* It is right to state that the idea of imitating Balfour's Committee of Imperial Defence for civil purposes was originally mooted by Lord Haldane as early as 1918.

The Labour Government of 1924, of which he was a member, considered the question further, apparently with economic rather than scientific enquiries in view; but nothing was done before they left office in the autumn of 1924. After the Labour Party returned to office in 1929, the organisation was modified in the way that Balfour had deprecated, and a standing committee was set up with the title of Economic Advisory Council. Whether this change will prove advantageous or permanent the future alone can show.

investigations are carried out by sub-committees in which a much larger latitude of choice is exercised, and there is as the backbone of the whole structure a permanent secretarial staff which serves the main committee and the sub-committees.

The above may seem somewhat of a digression, but its relevance will soon appear. Balfour's scheme of 1925 was closely copied from his scheme of 1904, and in fact it was possible to make use of the same offices and the same secretariat that already served the Cabinet and the Committee of Imperial Defence. Mr. Thomas Jones acted as principal secretary, with the assistance of Captain A. F. Hemming. The title chosen was the Committee of Civil Research.

Balfour was eager to make a beginning and to put the machinery thus created to the proof. The first subject of investigation was the Tsetse fly disease of Eastern and Central Africa. This had bearings both on health and on agriculture, and concerned the Colonial Office, the Fighting Services, the Foreign Office, and the Dominions. It was therefore typical of the kind of problem which the committee was created to deal with.

Balfour (acting for the Prime Minister) presided at the first meeting, and, as anticipated, a great lack of co-ordination was revealed. Under the Foreign Office many thousands a year were being spent in the Soudan, and spent to good advantage, particularly in the direction of quarantine. The Colonial Office representative admitted, under Balfour's cross-examination, that the Administration of Uganda knew nothing of this money being spent, and that they themselves had refused a trifling sum in respect of this kind of research. The man who was fighting the Tsetse fly in Uganda did not even know the man who was officially working at the same problem in the next colony.

The committee has remained active up to the time of writing. The Governor of Tanganyika who happened to be in England was later summoned to the committee, and as a result was glad to acknowledge the enlightenment it had given him, and to make consequent changes in his financial policy; and there were many other similar cases.

Balfour's influence did much to orient the policy of the Colonial Office towards the scientific aspect. This was the result of genuine conviction which he was able to impart, and not merely deference to superior authority. The machinery which he set up for the first time gave scientific men direct access to Ministers, without the intervention of lay officials, who were often unsympathetic to the scientific point of view, and deprived scientific advice of much of its effect.

Other scientific or semi-scientific subjects considered by the Committee of Civil Research during Balfour's regime (1925-29) were mineral content of natural pastures, Severn barrage, quinine supplies, dietetics, British pharmacopœia, research co-ordination, Kenya native welfare, geophysical surveying,

Great Barrier reef, mechanical transport, irrigation research, radium supplies, locust control.

The inauguration of the Committee of Civil Research was perhaps the last important achievement of Balfour's long career. The concluding phase of that career cannot be better summed up than in the words of one who was able to observe it at close quarters.\*

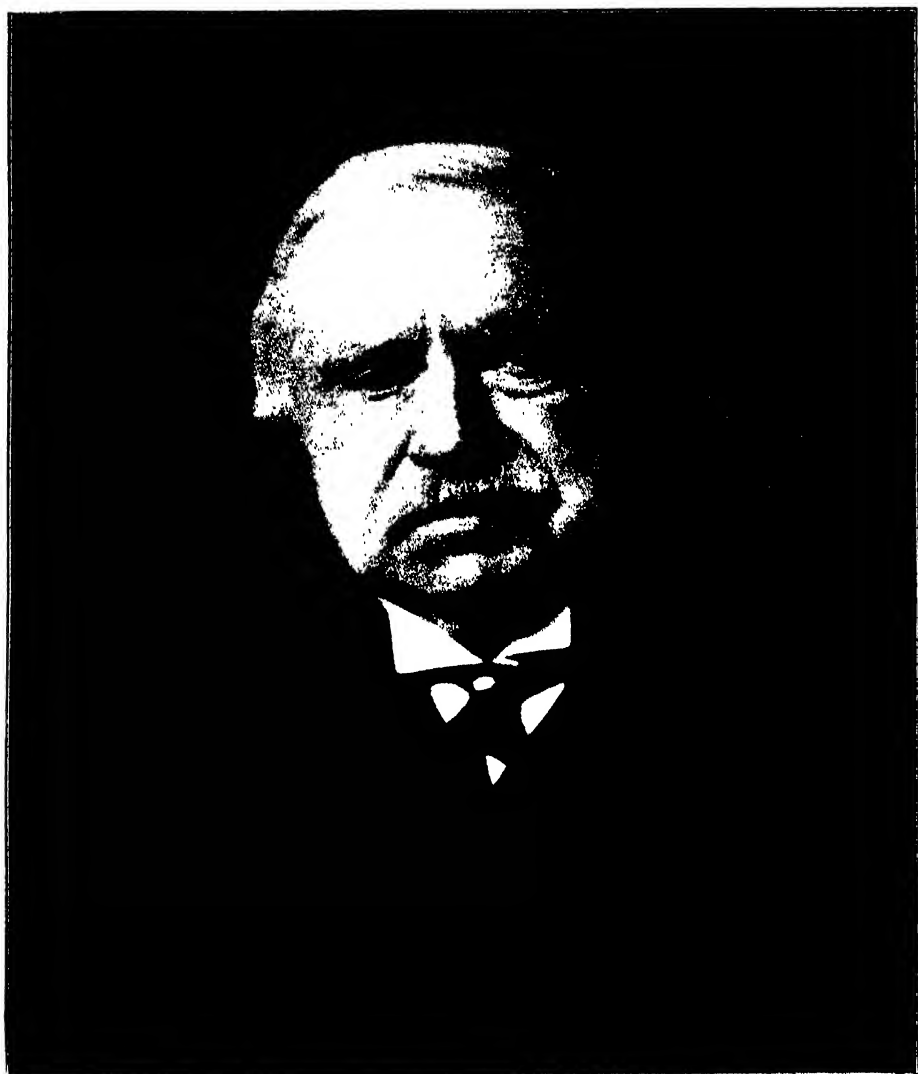
"The Lord Presidency used to be considered a general utility office. He converted it into a Ministry of research. The idea was not born in his fertile brain, for a committee of the Privy Council for Scientific and Industrial Research and a similar committee for Medical Research, had been established during the war, and Lord Haldane's committee on the machinery of government had recommended the creation of such a Ministry. But Lord Balfour it was who turned an experiment, which many thought destined to disappear with other war-time devices, into a reality which is now generally recognised as a permanent and essential part of modern government. His unparalleled prestige in the political and intellectual worlds, his liberation from the rough-and-tumble of party politics were favourable circumstances, but his abiding faith in the power of science to promote the happiness and well-being of man, his enthusiastic interest in the advance of knowledge, his sympathy with the scientific outlook and with young people, and his long experience of the way in which things have to be done in Great Britain, were the decisive factors."

RAYLEIGH.

---

\* Sir Frank Heath, G.B.E., K.C.B.





*W. Boyd Sander*

## SIR WILLIAM BOYD DAWKINS—1837–1929.

SIR WILLIAM BOYD DAWKINS, who died on January 15, 1929, was a versatile geologist, palæontologist, and anthropologist, who will be best remembered by his numerous important contributions to our knowledge of early man and the mammalia with which he was associated in Western Europe. He was actively engaged in research for more than half a century, and was a pioneer in modern methods and interpretations.

Dawkins was born at Buttington Vicarage, Welshpool, on December 26, 1837, the son of the Rev. Richard Dawkins. He was educated at Rossall School and Jesus College, Oxford, and graduated in 1860 with a first-class in natural science. For most of his time he devoted attention to classics, but he became deeply interested in geology through the influence of Professor John Phillips, and in 1861 he was the first recipient of the Burdett-Coutts scholarship which had just been founded to promote the study of geology. Immediately afterwards he was appointed to the Geological Survey, and for eight years he was occupied in surveying the Wealden and other formations in Kent and the Thames valley. At the same time he had experience of curatorial work in the Museum of Practical Geology, and in 1869, on the recommendation of Huxley, he left the Geological Survey to become curator of the Manchester Museum. He reorganised this museum as an adjunct to the Owens College, where he also lectured on geology; and in 1874, when the professorship of geology was separated from that of natural history, he was selected as first occupant of the chair. He was still professor of geology when the Owens College passed into the Victoria University of Manchester in 1880, and he retained the chair until his retirement in 1909.

Dawkins began his career of research in December, 1859, when he joined the Rev. J. Williamson in exploring Wookey Hole, a cavern near Wells, in Somerset. In describing the results in his first paper read to the Geological Society in 1862, he pointed out that during the later part of the Pleistocene period the cave had been occupied at times by hyænas, at other times by man. The implements of man and the remains of fires were clearly associated with the Pleistocene mammals. Henceforward Dawkins took part in many explorations of caves, and in 1874 he published his first book entitled "Cave Hunting: Researches on the evidence of caves respecting the Early Inhabitants of Europe." This book is both generally readable and full of original matter, partly culled from his scattered papers. Its concluding chapter is noteworthy as being the first elaborate attempt to classify the Pleistocene deposits by their fossil mammalia.

In 1875–78 Dawkins joined the Rev. J. Magens Mello in exploring caves in

Cresswell Crag, near Worksop, on the north-east border of Derbyshire. From a study of the mammalian remains and human implements and their distribution, he showed that there was a definite succession of faunas and human races. He proved that hippopotamus occurred only in the lowest layers, with the leptorhine rhinoceros and hyæna, but with neither mammoth nor reindeer and no traces of man. Then followed the mammoth, woolly rhinoceros, reindeer, and horse, with quartzite implements all rudely chipped. Finally, in the upper layers, he observed, without much change in the fauna, flint implements of the later palæolithic type, accompanied by implements of bone and antler and one piece of bone ornamented with the incised figure of the head of a horse. This was the first discovery of palæolithic cave art in England, and more examples have lately been found in the caves of the same district.

In 1903 Dawkins ended his special work on caves by discovering and interpreting a remarkable collection of Pliocene mammalian remains in a fissure in a quarry near Dove Holes, Derbyshire. He had already explained the absence of caves dating back to the Pliocene period by noting the great amount of denudation of the land-surface which had occurred since that period. He now, for the first time, identified a deeply-seated water channel into which some of the contents of a Pliocene cave had been washed and buried in mud. He recognised remains of mastodon, elephant, rhinoceros, horse, deer, *Machairodus*, and hyæna, all much water-worn but mostly sufficient to determine that they belonged to Upper Pliocene species. As there were marks of the teeth of hyænas on some of the bones, and as all the remains of mastodon belonged to young individuals, it was evident that these bones and teeth had been derived from a hyæna den.

Soon after he began research, Dawkins realised that the mammalian bones and teeth found in Pleistocene and later deposits needed more exhaustive study than had previously been devoted to them. He therefore visited all the collections which were available, and made notes and measurements to be incorporated in a valuable series of papers on these fossils. First, he distinguished the three Pleistocene species of rhinoceros by their dentition. Then he published papers on the British fossil oxen, and announced the discovery of part of the skull of a musk-ox (*Oribos*) in the brick-earth at Crayford, Kent. He also studied the fragmentary remains of deer, and attempted to define precisely the range of the mammoth in space and time. His most important work, however, was a volume on the cave-lion, which he prepared, in association with Mr. W. Ayshford Sanford, for the Palæontographical Society. He showed that this lion was identical with the existing *Felis leo*. At the same time he studied the cave-hyæna, and concluded that it could not be distinguished from the existing spotted hyæna, *H. crocuta*, of Africa. He eventually published a map, showing how Africa, Asia, and the Arctic regions all contributed a quota to the Pleistocene fauna of Western Europe.

While studying the Pleistocene mammals, Dawkins also paid some attention to those of earlier periods, especially the Pliocene. He pointed out that the deer, in the course of their evolution, acquired larger and increasingly complex antlers, until the maximum was reached at the beginning of the Pleistocene period; the successive changes in the antlers of the race were thus paralleled by the succession of the antlers during the individual life history of a modern deer, such as the common stag. In 1880 he reached a still more important generalisation, namely, that the Tertiary strata could be more satisfactorily classified by the stages in evolution exhibited by their mammalian fossils than by their fossil shells, which had been used by Lyell and his followers. Dawkins thus shared with Gaudry the beginning of the modern use of Tertiary mammals in stratigraphical geology.

As an illustration of the skill which Dawkins eventually acquired in identifying fragmentary mammalian fossils, may be specially mentioned his recognition in 1888 of the tooth of an Indian type of raccoon (*Ailurus*) in the Pliocene "Red Crag" of Suffolk. The complete dentition since found in rocks of the same age in Transylvania shows that the animal was very closely allied to *Ailurus*, only generically distinct.

Dawkins had already achieved his most important results in the study of fossil man by the year 1880, when he published an admirable summary in a volume entitled "Early Man in Britain and his Place in the Tertiary Period." This most readable work will always remain a classic, for it definitely marks an era in the progress of our knowledge. He recognised an early "River-drift Man" with roughly-chipped stone implements of nearly uniform type (now usually named Chellean and Acheulean), followed by a Cave Man with a little more skill in shaping stone to his uses (now named Mousterian) and a later Cave Man of superior ability, who not only shaped stone and bone implements but also ornamented them with drawings chiefly of animals of the chase (now named Aurignacian, Solutrean, and Magdalenian Man). He does not appear to have suspected that there might be extinct species, or even extinct genera, of intelligent Man; and so he concluded that Man was unlikely to date back to the Pliocene period when there were no existing species among the other mammals. He compared the art of the latest Cave Man with that of the existing Eskimo, and he identified among the bone implements an arrow-straightener like that used by the Eskimo. He therefore thought that there were no descendants of the Cave Man now in Europe, and agreed with certain French and Belgian geologists that this man had retreated with the reindeer and the musk-ox to the Arctic region—a conclusion which has not been altogether substantiated, though the Chancelade skeleton of a Cave Man found near Périgueux, in the Dordogne, is generally recognised as closely similar to that of an Eskimo.

In his later years, Dawkins paid increasing attention to economic geology,



and he was much engaged in problems of water supply and engineering. Among other undertakings in which he was concerned was that of the Channel Tunnel proposed by Sir Edward Watkin and his associates. Dawkins suggested that the shaft which had been sunk at the Dover end of the projected tunnel workings would be a most suitable spot for a boring to find the buried coal-field which many geologists had already concluded must underlie south-east England. The boring was actually made, and in 1890 productive coal measures were reached at a depth of 1100 feet. The exploitation of the Kent coalfield was thus begun.

As curator of the Manchester Museum and as a citizen of Manchester, Dawkins took a most prominent part in city life. He was a fascinating popular lecturer, and his numerous talks at the Museum, which were continued even after his retirement, were a highly appreciated feature in the intellectual life of the community. Shortly before his death he gave to the city a collection of photographs, drawings, and plaster casts to illustrate the dawn of art in Europe. On the other hand, he was very frequently absent from Manchester on his various enterprises, and he kept touch especially with Oxford and London. At Oxford he had been contemporary with John Richard Green, the historian, with whom he formed a life-long friendship. He was made an honorary Fellow of his college in 1882, and was admitted to the honorary degree of D.Sc. in 1900. He was elected a Fellow of the Geological Society in 1861, and received the Lyell Medal from that Society in 1889, the Prestwich Medal in 1918. He was elected a Fellow of the Royal Society in 1867, and served on the Council in 1889-91. He was knighted in 1919.

In 1866 Dawkins married Miss Frances Evans, who died in 1921, leaving a daughter. In 1922 he married the widow of Mr. Hubert Congreve, M.I.C.E., and she survived him.

A. S. W.





JOHN WILLIAM EVANS.

## JOHN WILLIAM EVANS—1857–1930.

JOHN WILLIAM EVANS was born in 1857 and died on November 16, 1930. Although educated with a view to a legal career, called to the Bar in 1878 and taking the LL.B. degree in the University of London, he succumbed to the attractions of science and became a student of geology under Judd at the Royal College of Science. His course was crowned by the award of the Murchison Medal in 1889, and by his appointment to a demonstratorship in that department, which he held till, in 1891, he made his first journey to South America as geologist on an expedition to the upper reaches of the River Paraguay and the Tapirapuam in Brazil.

Shortly after his return he was appointed State Geologist to Junagarh (Kathiawar), but after a year he passed to Mysore, where he soon became Chief Geologist and Chief Inspector of Mines and Explosives. In India, while his professional work was chiefly on the economic side, he made important discoveries on the origin of certain limestones, showing that wind and other mechanical agencies had been in operation, as, he concluded, had also been the case with some of the oolitic limestones in Britain and elsewhere. He also studied some remarkable igneous rocks, and made large collections of others, some of which formed the subject of laboratory investigations by his advanced students under his careful and inspiring supervision. After his return from India, and the publication of his more important scientific observations, the Geological Society awarded him its Lyell Fund, and the University the degree of D.Sc.

He was next appointed leader of a scientific expedition to Caupolicán Bolivia, which took him high up into the Andes and back by the Madeira cataracts and the Amazon. The 'Geographical Journal' published the accounts of his American explorations, and his study of the hydrography of the Andes, while his geological papers included observations on the formation of breccias in the courses of the Andean rivers and an account of the geology of Matto Grosso (Brazil), a district then little known.

In 1904 Evans became a Special Assistant at the Imperial Institute, and later he served on the Colonial Survey Committee and the Mineral Council of this body. Most of the mineral material sent to the Institute, and for its museum, passed through his hands for determination and evaluation, giving him an opportunity for acquiring a wide knowledge of the mineral resources of the Colonies and Dependencies, of which he was not slow to avail himself. He also gained an acquaintance with the work and results of the Colonial Geological Surveys to which he gave warm sympathy and active support.

throughout his life. Later on he was made a Governor of the Imperial Mineral Resources Bureau, when that was established, and for several years was the official representative of the Colonial Office on it. He took a keen interest in its work, particularly in advocating the expert examination, or more extensive geological survey, of those parts of the Empire of which the mineral wealth was little known. He also placed his legal knowledge at the disposal of those who were modifying or codifying the laws relating to mining or exploitation in the various Dependencies.

From 1918 to 1922 Evans was Adviser to the Colonial Office on minerals other than coal and oil, and he sat on the Geological Survey section of the Colonial Survey Committee from its institution in 1912 till his death. When the Imperial Mineral Resources Bureau was amalgamated with the Imperial Institute he became one of the Institute Advisory Council on Mineral Resources, and remained a member till the end of his life.

Meanwhile he served on the staff of the Birkbeck College as Lecturer in Geology from 1906 till 1920, and was Lecturer in Petrology at the Imperial College from 1912 till his retirement on the age limit in 1927. As a teacher he was clear-headed and inspiring in his class work, but even more efficient in his handling of post-graduate students, many of whom became geological surveyors, teachers, or investigators of uncommon merit. His students are or have been carrying on valuable research in India, Australia, New Zealand, Africa, and other parts of the Empire, as well as at home.

Evans's own researches cover so wide a field that it is not easy to give a connected account of them. His chief interests were with mineralogy and petrology. He made many contributions to the 'Mineralogical Magazine' and other journals, in most of which he suggested improvements in existing methods of investigation; he designed new forms of the quartz-wedge and other devices for determining the optical constants of minerals, advocated the use of the gnomonic projection in crystallography, and criticised and revised the usual classifications, nomenclature, and notation of crystals and minerals. While well acquainted with the normal methods and results of descriptive petrology, and using them in his papers, his interests were always with larger questions of genesis; the origin and relations of the alkali rocks, the American system of classification and the light thrown by it on theoretical questions, and the attempted introduction into this country of geochemical research, somewhat on the lines of the Geophysical Institute of Washington. Some of the results of his work were gathered up in his own book on the determination of minerals in thin sections under the microscope, and in that by himself and Mr. Macdonald Davies on Crystallography.

Another important piece of work which Evans started while a student at the Royal College was on the Old Red Sandstone of Caithness, lacustrine rocks, which it was difficult to place in their correct position in the sequence. This

was taken up again in his later years, but this time among the equivalents, then thought to be wholly marine, in Devon. He helped much to clear away difficulties in the stratigraphy of these rocks, made important discoveries of fossils and other structures which effected linkages between them and their northern representatives, discovered lacustrine horizons among them, and criticised and improved the correlation of British and Continental Devonian rocks. He contributed a chapter on the Devonian to the British volume of 'Regionale Geologie,' of which he was editor. This work, published in Germany in the early years of the war, has now appeared in a thoroughly revised form in England, also under Dr. Evans's editorship, with the collaboration of Dr. Stubblefield.

During the great war Dr. Evans was gazetted Lieut.-Colonel, placed in charge of a section of the northern defences of London, and received the Volunteer Decoration. He was also made a J.P., and served on one of the army tribunals. At the close of the war he became President of Section C (Geology) of the British Association at Bournemouth, and of the National Union of Scientific Workers.

Evans directed many excursions of the Geologists' Association and kindred bodies to the scenes of his own research, Devon and near London, and also visits of instruction to the museum of the Imperial Institute, and the mineral sides of several London exhibitions. His work for the Geologists' Association culminated in 1912, when he was elected President. His two addresses dealt with the "Wearing down of the rocks." His own mathematical ability enabled him to treat the work of ice, water, and wind, in a novel fashion, and the addresses were full of his own observations in many parts of the world, and of others gathered from his extensive reading.

Although he communicated many papers to the Geological Society some of his most valuable contributions were, perhaps, made in the discussions, at which he was a frequent and welcome speaker, chiefly because of the fresh and unexpected light that he often threw upon subjects under discussion. The Society awarded him its Murchison Medal in 1922, and in the following year he received the C.B.E. He was elected a Fellow of the Royal Society in 1919. He was President of the Geological Society for two years, 1924 to 1926, his two annual addresses dealing with areas of tension and of compression in the earth-crust. He collected a vast number of instances to illustrate these phenomena and drew valuable conclusions from them. About this time too he eagerly took up the hypothesis of Wegener, opening a joint discussion of the Geographical and Geological Sections of the British Association on it, and afterwards revising Skerl's translation of Wegener's book into English.

On retiring from his teaching work Evans became Chairman of the Geophysical Company, and on its behalf visited many parts of the Near East, including Egypt and Palestine, in order to test the applicability of geophysical

methods, and especially sound-ranging, to the elucidation of geological structure. This work was especially congenial to him, as it gave him excellent opportunities of studying the geology of areas new to him and brought him into intimate association with those whose original observations and descriptions were the main sources of knowledge in them.

Evans rarely missed an opportunity to travel, either in Europe or farther afield. He attended the International Geological Congresses in Brussels in 1922, in Madrid in 1926, and in Pretoria in 1929. The last was of particular interest to him as he had been collecting and correlating for a geological map of Africa, which it was hoped would be published by the Congress, the British material supplied by the various Colonial and Dominion Surveys. At the end of the South African Congress he journeyed from the Cape to Cairo, taking the opportunity to visit and study the ground under the charge of the several Geological Surveys, on some of which were old students of the Imperial College in whose training he had taken a prominent part. Shortly before his death he had arranged to prepare a report on St. Helena for the Colonial Development Advisory Committee.

After his return in the spring of 1930 Evans visited the Continent, and was planning another journey to the East when death overtook him. In him we have lost a mind of intense activity, hungry for new facts, and with a remarkable faculty for co-ordinating them. Though perhaps it cannot be said that he completed any very great and outstanding piece of work, he ranged over the whole science of geology and not a few of its allied sciences, always revealing fresh points of view, and always discovering new facts or bringing old ones into new relations. Further, through his friendships, his pupils, his well known public spirit, which placed the public good always before his own private interests, and the wise operation of his insight and control on the bodies or societies that he served, he exercised a far-reaching influence for good in the science that he loved.

W. W. W.

## INDEX to VOL. CVII. (B)

- Anterior pituitary body (Hill and Parkes), 455.  
 Anterior pituitary body and gonads (Parkes), 30, 39.  
 Askew (F. A.), Bourdillon (R. B.), Bruce (H. M.), Jenkins (R. G. C.) and Webster (T. A.)  
     The Distillation of Vitamin D, 76 ; The Effects of Further Irradiation of the Radiation  
     Products of Ergosterol, 91.  
 Bacteriophage, theories (Bordet), 398.  
 Balfour (Earl of), obituary, iv.  
 Bordet (J.) The Theories of the Bacteriophage, 398.  
 Bourdillon (R. B.) See Askew and others.  
 Briggs (G. E.) The Accumulation of Electrolytes in Plant Cells, 248.  
 Bruce (H. M.) See Askew and others.  
 Cell structure and activity (Penrose and Quastel), 168.  
 Cellular individuality in the higher animals (Todd), 197.  
 Charles (E.) Metabolic Changes associated with Pigmentary Effector Activity and Pituitary  
     Removal in *Xenopus laevis*, I, II, 486, 504.  
 Ciliary movement, mechanism (Gray), 313.  
 Component c of cytochrome (Hill and Keilin), 286.  
 Corpus luteum, functions (Parkes), IV, 188.  
 Corpus luteum of the mouse and rabbit (Deanesly), 60.  
 Croonian Lecture. See Bordet.  
 Crowther (E. M.) The Relationship of Climatic and Geological Factors to the Composition  
     of Soil Clay and the Distribution of Soil Types, 1.  
 Dale (A.) See Endres and others.  
 Darlington (C. D.) A Cytological Demonstration of "Genetic" Crossing-Over, 50.  
 Dawkins (Sir William Boyd), obituary, xxiii.  
 Deanesly (R.) The Development and Vascularisation of the Corpus Luteum in the Mouse  
     and Rabbit, 60.  
 Duke-Elder (P. M.) See Duke-Elder and Duke-Elder.  
 Duke-Elder (W. S.) and Duke-Elder (P. M.) The Contraction of the Extrinsic Muscles of  
     the Eye by Choline and Nicotine, 332.  
 Eccles (J.) Studies on the Flexor Reflex, III, 557.  
 Eccles (J.) and Sherrington (Sir Charles) Studies on the Flexor Reflex, I, II, IV, V, 511,  
     535, 586, 597.  
 Echinoderm oogenesis (Harvey), 417.  
 Electrolytes in plant cells (Briggs), 248.  
 Endres (G.), Matthews (B. H. C.), Taylor (H.) and Dale (A.) Observations on Certain  
     Physiological Processes of the Marmot, I.-V., 222, 231, 241.  
 Ergosterol, radiation products (Askew and others), 91.  
 Evans (J. W.), obituary, xxvii.  
 Extrinsic muscles of the eye, contraction (Duke-Elder and Duke-Elder), 332.



- Fernando (W.) The Origin of the Mesoderm in the Gastropod *Viviparus*, 381 ; The Origin and Development of the Pericardium and Kidneys in *Ostrea*, 391.
- Flexor reflex, studies (Eccles), III, 557.
- Flexor reflex, studies (Eccles and Sherrington), I, II, IV, V, 511, 535, 586, 597.
- Gadow (H. F.), obituary, i.
- Geiger (A.) The Isolation by Cataphoresis of two different Oxyhaemoglobins from the Blood of some Animals, 368.
- "Genetic" crossing-over (Darlington), 50.
- Gray (J.) The Mechanism of Ciliary Movement, VI, 313.
- Harvey (L. A.) Studies on Echinoderm Oogenesis, I, II, 417, 441.
- Heat of rigor of mammalian muscle (Smith), 214.
- Heat production in isometric and isotonic twitches (Hill), 115.
- Hill (A. V.) The Heat Production in Isometric and Isotonic Twitches, 115.
- Hill (R.) A Method for the Estimation of Iron in Biological Material, 205.
- Hill (R.) and Keilin (D.) The Porphyrin of Component c of Cytochrome and its Relationship to other Porphyrins, 286.
- Hill (M.) and Parkes (A. S.) The Relation between the Anterior Pituitary Body and the Gonads, I, II, III, 30, 39, 455.
- Iron, estimation in biological material (Hill), 205.
- Jenkins (R. G. C.) See Askew and others.
- Keilin (D.) See Hill and Keilin.
- Krishnamurti (K.) The Scattering of Light in Protein Solutions (Abstract), 269.
- Ludford (R. J.) The Vital Staining of Normal and Malignant Cells, III, 101.
- Margaria (R.) The Osmotic Changes in Some Marine Animals, 606.
- Marmot, physiological processes (Endres and others), 222, 231, 241.
- Matthews (B. H. C.) See Endres and others.
- Mayneord (W. V.) Secondary Electronic Emissions from Metal Foils and Animal Tissues (Abstract), 344.
- Mellanby (J.) Prothrombase—Its Preparation and Properties, 271.
- Mesoderm, origin in *Viviparus* (Fernando), 381.
- Metabolic changes in *Xenopus laevis* (Charles), 486, 504.
- Moppett (W.) The Differential Action of X-Rays on Tissue, Growth and Vitality, II, III, IV, 293, 302, 308.
- Moran (T.) The Frozen State in Mammalian Muscle, 182.
- Muscle, mammalian, frozen state (Moran), 182.
- Obituary notices :—
- |                                   |                      |
|-----------------------------------|----------------------|
| Balfour, Earl of, iv.             | Evans, J. W., xxvii. |
| Dawkins, Sir William Boyd, xxiii. | Gadow, H. F., i.     |
- Oogenesis, echinoderm (Harvey), 417.
- Osmotic changes in marine animals (Margaria), 606.

*Pericardium*, origin and development of pericardium and kidneys (Fernando), 391.  
*Oxyhaemoglobins*, isolation by cataphoresis (Geiger), 368.

*Parkes* (A. S.) See also Hill and Parkes.

*Parkes* (A. S.) The Functions of the Corpus Luteum, IV, 188.

Penrose (M.) and Quastel (J. H.) Cell Structure and Cell Activity, 168.

Pericardium, origin and development (Fernando), 381.

Pincus (G.) Observations on the Living Eggs of the Rabbit, 132.

Plant cells, accumulation of electrolytes (Briggs), 248.

Porphyrin of Component c of Cytochrome (Hill and Keilin), 286.

Presidential Address, 348.

Prothrombase, preparation and properties (Mellanby), 271.

Pupillary mechanism of teleostean fish (Young), 464.

Quastel (J. H.) See Penrose and Quastel.

Rabbit, living eggs (Pincus), 132.

Red blood corpuscle, individuality (Todd), 197.

Rigor of mammalian muscle (Smith), 214.

Rutherford (Sir Ernest) Address at Anniversary Meeting, 348.

Sherrington (Sir Charles) See Eccles and Sherrington.

Smith (E. C.) The Heat of Rigor of Mammalian Muscle, 214.

Soil clay, relationship of climatic and geological factors (Crowther), 1.

Soil types, distribution (Crowther), 1.

Staining of normal and malignant cells (Ludford), 101.

Taylor (H.) See Eudres and others.

Todd (C.) Cellular Individuality in the Higher Animals, with special reference to the Individuality of the Red Blood Corpuscle, II, 197.

Twitches, isometric and isotonic (Hill), 115.

*Uranoscopus scaber*, pupillary mechanism (Young), 464.

Vital staining of normal and malignant cells (Ludford), 101.

Vitamin D, distillation (Askew and others), 76.

*Viviparus*, origin of mesoderm (Fernando), 381.

Webster (T. A.) See Askew and others.

X-Rays, differential action on tissue, growth and vitality (Moppett), 293, 302, 308.

Young (J. Z.) The Pupillary Mechanism of the Teleostean Fish, *Uranoscopus scaber*, 464.



